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EXCRETION OF ADMINISTERED RADIOCALCIUM[1] FOLLOWING THYROPARATHYROIDECTOMY, OR BILATERAL NEPHRECTOMY, AND THE INJECTION OF PARATHYROID EXTRACT[2]

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PREVIOUS reports (Thomson and Collip, 1932; Greep, 1948) have indicated that the urinary excretion of calcium is reduced and the fecal excretion diminished, or slightly elevated, after parathyroidectomy or thyroparathyroidectomy, and that both the urinary and fecal excretion of calcium are increased by the administration of parathyroid extract.

The results of the present experiments in conjunction with the results of previous investigations (Tweedy and Campbell, 1944; Tweedy, Chilcote, and Patras, 1947) suggest that parathyroid hormone affects the excretion of calcium through its action on the phosphorus metabolism.

MATERIALS AND METHODS

Seventy-six rats of the Sprague-Dawley strain were used. The stock diet, Purina Laboratory Chow, on which they were reared contained approximately 1.18% of calcium and 0.98% of phosphorus.

Two radioactive calcium preparations were available during the course of the experiments. The less active of the two preparations was obtained in the form of calcium carbonate with a specific activity of only 0.3 millicurie per gram. The required amount of this preparation was converted to calcium chloride and the aqueous solution adjusted to a pH of 5.9. Of this solution 2 ml., containing 7 mg. of calcium and 5 microcuries

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(310×10^{-9} mg.) of radio-calcium, constituted the dose injected into each of the animals shown in Table 1. The other radioactive calcium preparation, used in the experiments, was obtained in the form of calcium chloride of high specific activity and essentially carrier-free. The required amount of the latter preparation to give the desired number of microcuries of radiocalcium was used to label the dose of calcium chloride received by each of the animals shown in Tables 2 to 5. The labeled calcium was administered subcutaneously (Tables 1, 2, and 4) or intraperitoneally (Tables 3 and 5). Parathyroid extract (Lilly), when used, was injected subcutaneously.

After the injections the animals were placed in separate wire metabolism cages over

TABLE 1. EXCRETION OF ADMINISTERED RADIOCALCIUM DURING AN 18-HOUR PERIOD ON VARIOUS DAYS AFTER THYROPARATHYROIDECTOMY

Group[1]	No. of rats	Body weight (gms.)	Time after operation (days)	Serum Calcium (mg. %)	Per cent[2] of administered radiocalcium excreted	
					Urine	Feces
1. Experimental	8	205 ± 54	1–8	6.30 ± 1.30	2.67 ± 2.04	6.56 ± 1.74
1. Control	8	207 ± 31		9.70 ± 0.35	2.73 ± 2.15	9.76 ± 3.35
2. Experimental	8	216 ± 29	9–15	6.30 ± 1.39	2.42 ± 1.52	7.94 ± 4.80
2. Control	8	219 ± 26		9.69 ± 0.57	2.71 ± 2.10	8.50 ± 3.33
3. Experimental	5	268 ± 14	24–40	9.10 ± 0.90	5.80 ± 2.51	9.32 ± 2.30
3. Control	5	254 ± 20		10.40 ± 0.30	5.52 ± 2.30	9.90 ± 2.60

[1] Each experimental animal and its control in Groups 1, 2, and 3 received a subcutaneous injection of 7 mg. of calcium, containing 5 microcuries of radiocalcium. One hour after the administration of the first dose, the animals of Group 3 received an additional dose of 7 mg. of labeled calcium. The controls were subjected to no operative procedures.

[2] Mean per cent. The measure of variability is indicated by the standard deviation from the mean.

urine-feces separators, and supplied with water and the stock diet. At the end of the experimental period, blood samples were collected from the cut end of the tail, and the animals dispatched by a blow on the head. The cage washings were added to the urine, and the contents of the gastrointestinal tract were added to the last feces which were collected. In those instances in which attempts were made to recover the entire dose of radiocalcium that had been injected, the viscera were removed and the carcass autoclaved at 10–15 lbs. pressure for 10 minutes in order to facilitate the separation of the skeleton and the soft tissues. The methods used for the preparation of the tissues and excreta for the measurement of radioactivity, and the counting technique, are described in a previous paper (L'Heureux, Tweedy, and Zorn, 1949). Serum calcium determinations were made by the method of Kramer and Tisdall (1921) as modified by Tweedy and Koch (1929).

EXPERIMENTAL PROCEDURES AND RESULTS

Effect of thyroparathyroidectomy upon the excretion of injected radiocalcium: Table 1 presents in summarized form the terminal serum calcium values and the percentages of the dose of radiocalcium recovered in the excreta of several thyroparathyroidectomized rats, and their controls, at the end of an 18-hour period on various days after the experimental

animals had been operated on. Not shown in Table 1 is a group of 4 thyroparathyroidectomized rats and their 4 controls. Six hours after these experimental animals had been operated on both they and their controls received a subcutaneous injection of 7 mg. of calcium, containing 5 microcuries of radiocalcium. Assays of the urine and the feces from each rat during the next 8 days revealed that during the 8-day period the animals of the experimental group had excreted $2.51 \pm 1.32\%$ of the administered radiocalcium in the urine and $16.79 \pm 6.89\%$ in the feces, while the controls excreted by these routes $2.45 \pm 0.73\%$ and $22.10 \pm 8.85\%$, respectively.

TABLE 2. DISTRIBUTION, RETENTION, AND EXCRETION OF RADIOCALCIUM[1] TWENTY-FOUR HOURS AFTER THYROPARATHYROIDECTOMY AND EIGHTEEN HOURS AFTER ADMINISTRATION

Tissues	Experimental animal Body wt. 188 gms.		Control Body wt. 186 gms.	
	Ash wt. (gms.)	Per cent of dose recovered	Ash wt. (gms.)	Per cent of dose recovered
Bones	3.89	68.22	3.84	64.84
Skin and hair	0.24	0.37	0.32	0.66
Spleen, liver, kidneys, lungs and heart	0.25	0.37	0.45	0.54
Stomach and intestines	0.28	0.56	0.35	0.67
Residual carcass	1.30	11.69	1.51	7.07
Excreta:				
Urine	0.40	3.98	0.79	6.13
Feces	0.70	6.12	1.38	10.51
Per cent of dose recovered		91.31		90.42

[1] Each animal was injected subcutaneously with 2.5 mg. of calcium, containing 10 microcuries of radiocalcium.

The experimental animals showed serum calcium values of $7.21 \pm 1.25\%$ at the end of the experiment as compared with serum calcium values of $9.69 \pm 0.57\%$ shown by the controls.

Effect of thyroparathyroidectomy upon the distribution, retention, and excretion of injected radiocalcium: Six hours after a rat had been thyroparathyroidectomized both it and the control were injected with 2.5 mg. of calcium, containing approximately 10 microcuries of radiocalcium. Eighteen hours later blood was drawn from each animal, and the animals sacrificed. The terminal serum calcium values shown by the experimental animal and the control were 6.0 mg.% and 10.8 mg.%, respectively. The results of the assays of the tissues and excreta for radiocalcium are presented in Table 2.

Effect of parathyroid extract upon the distribution, retention, and excretion of administered radiocalcium after thyroparathyroidectomy: Each of 4 rats

TABLE 3. EFFECT OF PARATHYROID EXTRACT UPON THE DISTRIBUTION, RETENTION, AND EXCRETION OF RADIOCALCIUM INJECTED AFTER THYROPARATHYROIDECTOMY

Group	No. of rats	Body wt. (gms.)	Serum calcium (mg. %)	Per cent[1] of injected radiocalcium recovered per gm.				Per cent[2] of radiocalcium recovered	
				Muscle	Liver	Kidney	Femur	Urine	Feces
Experimental animals[1]	4	205 ± 9	13.8 ±0.4	0.041 ±0.003	0.028 ±0.007	0.081 ±0.019	3.23 ±0.56	1.22 ±0.58	12.18 ±1.91
Controls	4	202 ±10	10.6 ±0.8	0.040 ±0.013	0.019 ±0.006	0.048 ±0.004	3.70 ±0.44	1.19 ±0.70	16.20 ±1.91

[1] Each experimental animal received 500 units of parathyroid extract 24 hours after thyroparathyroidectomy. One hour after the administration of parathyroid extract, each experimental and its control was injected intraperitoneally with 0.7 mg. of calcium, containing 5 microcuries of radiocalcium.
[2] Mean per cent. The measure of variability is indicated by the standard deviation from the mean.

received 500 units of parathyroid extract 1 day after extirpation of the thyroid and parathyroids. One hour after the injection of parathyroid extract, each of these animals and its respective control received an intraperitoneal injection of 0.7 mg. of calcium, containing 5 microcuries of radiocalcium. Eighteen hours later they were sacrificed. The results of the assays of the tissues and excreta for radio-calcium are shown in Table 3.

Effect of parathyroid extract upon the excretion of administered radiocalcium by normal rats: Each of 4 rats was injected with 500 units of parathyroid extract, and 18 hours later each animal received an additional 250 units. One lour after the last dose, each experimental animal and its

TABLE 4. EFFECT OF PARATHYROID EXTRACT UPON THE EXCRETION OF INJECTED RADIOCALCIUM BY NORMAL RATS

Exp. rat[1] No.	Body wt. (gms.)	Hours after injection of radiocalcium[2]	Per cent of injected radiocalcium excreted		Control No.	Body wt. (gms.)	Per cent of injected radiocalcium excreted	
			Urine	Feces			Urine	Feces
1	327	18	2.73	2.52	1	292	0.40	8.80
		42	0.27	0.35			0.16	3.87
		66	0.20	2.04			0.08	1.23
2	309	18	4.40	6.64	2	323	1.30	9.67
		42	2.93	8.38			0.70	9.98
		66	2.20	3.00			0.42	5.17
3	291	18	2.69	3.30	3	292	0.26	11.20
		42	0.52	0.24			0.14	3.27
		66	0.28	1.30			0.09	1.38
4[3]	262	18	3.18	1.10	4	269	0.47	8.50
		42	1.25				0.19	3.25
		66					0.09	1.24

[1] Each experimental animal was injected subcutaneously with 500 units of parathyroid extract. Eighteen hours later an additional 250 units were injected subcutaneously.
[2] One hour after the administration of the second dose of parathyroid extract, each experimental animal and its control was injected subcutaneously with a dose of 0.21 mg. of calcium, containing 5 microcuries of radiocalcium.
[3] Found dead in cage approximately 50 hours after the experiment was begun.

control received a dose of 0.21 mg. of calcium, containing 5 microcuries of radiocalcium. Sixty-six hours later the animals were sacrificed. The terminal serum calcium values of experimental animals 1, 2, and 3, Table 4, were 11.0, 10.6, and 10.3 mg.%, respectively, while those of their respective controls were 9.7, 10.1, and 10.3 mg.%. One experimental animal died approximately 50 hours after the experiment was started; hence, no analysis was made of its serum calcium. The fractions of the administered radiocalcium recovered in the excreta of the animals are shown in Table 4.

Distribution, retention, and excretion of radiocalcium after bilateral nephrectomy and the injection of parathyroid extract: Ten rats were subjected

TABLE 5. DISTRIBUTION, RETENTION, AND EXCRETION OF ADMINISTERED RADIOCALCIUM AFTER BILATERAL NEPHRECTOMY AND THE INJECTION OF PARATHYROID EXTRACT

No. of groups	Animals[1]	Body weight (gm.)	Dose of labeled calcium[2] (mg.)	Per cent* of dose recovered per gram				Per cent* of dose recovered		
				Blood	Liver	Muscle	Femurs	Stomach and intestines	Feces	Urine
3	A	320± 12	0.41	0.024± 0.002	0.019± 0.004	0.024± 0.006	3.40± 0.20	0.69± 0.02	6.03± 1.43	
	B	314± 17	0.41	0.023± 0.005	0.016± 0.001	0.023± 0.002	2.94± 0.18	0.64± 0.04	8.44± 0.96	
	C	340± 5	0.41	0.018± 0.002	0.014± 0.002	0.017± 0.005	3.00± 0.10	0.41± 0.04	10.92± 1.44	0.91± 0.70
1	A	318	1.75	0.100	0.048	0.054	1.16	1.40	12.20	
	B	312	1.75	0.112	0.041	0.060	0.65	1.96	15.20	
	C	336	1.75	0.064	0.035	0.056	0.58	1.16	25.00	7.04
1	A	181	2.50	0.027	0.015	0.421	2.44	0.44	15.66	
	B	175	2.50	0.031	0.022	0.192	2.46	0.49	11.82	
	C	184	2.50	0.011	0.006	0.018	1.91	0.27	9.67	9.92

[1] A represents the experimental animal, B the nephrectomized control, and C the normal control.
The rats designated by the letter A were each injected subcutaneously with 500 units of parathyroid extract 4 hours after they were completely nephrectomized. One hour after the injection of parathyroid extract each rat (A, B, and C) was injected intraperitoneally with the designated dose of calcium, containing 10 microcuries of radiocalcium. Eighteen hours after the injection of the labeled calcium the animals were sacrificed.
* Mean per cent. The measure of variability for three values is indicated by the standard deviation from the mean.

to unilateral nephrectomy and 15 days later the remaining kidney was removed from each animal. Four hours after the second operation 5 of the bilaterally nephrectomized rats received a single subcutaneous injection of 500 units of parathyroid extract. One hour later the hormone-treated nephrectomized rats and their nephrectomized and normal controls were injected intraperitoneally with a dose of calcium containing 10 microcuries of radiocalcium. The results are presented in Table 5.

DISCUSSION

After thyroparathyroidectomy (Table 1), or after parathyroidectomy alone (Tweedy and Chandler, 1929), the serum calcium has been observed to fall to low values (5–7 mg.%) within the first 24 to 48 hours, but return to the lower limits of the normal range (9–12 mg.%) after several days. Injection of labeled calcium soon after the removal of the thyroid and

parathyroids revealed a decrease in the fecal excretion of administered radiocalcium, but no consistent change in the urinary excretion. These results were clearly evident in the case of those animals (Table 1) which were injected with labeled calcium within the first 8 days after extirpation of the thyroid and parathyroids.

Eight days after the surgical interventions, the experimental animals (Table 1) showed a tendency to excrete larger amounts of radiocalcium in the feces, and 24 to 40 days after the animals had been operated on the fraction of the injected radiocalcium recovered in the feces of the experimental animals was approximately equal to the fraction recovered in the feces of the controls. There was one animal, however, which did not conform to this trend. This animal, which is not included in Table 1, was in moderate tetany 33 days after thyroparathyroidectomy at the time that an 18-hour excretion text has been completed. It was found to have excreted 1.5% of a dose of 7 mg. of labeled calcium in the urine and 5.6% in the feces, while the control excreted by these routes 4.2% and 9.7%, respectively. The terminal serum calcium values of this experimental animal and its control were 5.7 mg.% and 9.5 mg.%, respectively. Undoubtedly, the high calcium content (1.18%) of the diet, on which our thyroparathyroidectomized rats were maintained, contributed to their ability to remain free of manifest tetany. Shelling and Ascher (1932) found in their studies that rats deprived of their parathyroids rarely exhibited more than slight signs of tetany unless placed on a low calcium-high phosphorus diet, in which event tetany of varying severity developed in nearly 100% of the animals.

In contrast to its effect upon the excretion of radiocalcium, thyroparathyroidectomy in rats has been shown (Tweedy, Chilcote, and Patras, 1947) to cause a marked decrease in the urinary excretion of radiophosphorus, but no consistent change in the fecal excretion. The retained radiophosphorus was found to have accumulated in both the soft tissues and the bones. The accumulation of phosphorus in the tissues of the thyroparathyroidectomized dog and this animal's inability to excrete the phosphorus was shown by Greenwald (1911) to be due to the removal of the parathyroid glands, and not to the concurrent thyroidectomy. The manner in which the thyroid affects calcium metabolism is still unsettled, but on the basis of work during past few years it has been suggested (Greep, 1948) that the effect of the thyroid on calcium metabolism may be mediated through the parathyroids.

The distribution of radiocalcium in the tissues of one of our animals (Table 2), in which the serum calcium had dropped to 6 mg.% 24 hours after the removal of the thyroid and parathyroids, indicates a definite increase in the retention of the extraskeletal calcium but only a small

overall increase in the retention of the skeletal calcium. It seems probable that the decreased fecal excretion of injected radiocalcium, as well as the fall in serum calcium, seen in our thyroparathyroidectomized rats (Tables 1–2), was due to the retention of a fraction of the mobilizable calcium by the accumulated phosphorus.

It will be noted (Table 3) that the fraction of the administered radiocalcium recovered in the feces of the thyroparathyroidectomized rats after the injection of 500 units of parathyroid extract was smaller than the control value, and that an increase in the renal excretion of radiocalcium was not detected despite an elevation of the serum calcium level and a marked accumulation of radiocalcium in the kidneys. Since the injection of only 5 units of parathyroid extract in thyroparathyroidectomized rats has appeared to produce an immediate increase in the urinary excretion of injected radiophosphorus (Tweedy, Chilcote, and Patras, 1947), it seems probable that under the influence of 500 units the radiocalcium accumulated in the kidneys of the experimental animals (Table 3) prior to the elevation of the serum calcium level, and that it was caused by an increased flow of inorganic phosphate to the kidneys that coincided with the appearance of the injected radiocalcium in the blood. In a previous investigation (Tweedy, 1945), it was shown that radiostrontium, whose metabolism closely resembles that of radiocalcium (Pecher, 1942), accumulated in the kidneys of normal rats which had been injected with inorganic phosphate shortly before or after the injection of radiostrontium.

In the study just referred to (Tweedy, 1945), it was also observed that the injection of parathyroid extract in normal rats had no effect upon the excretion of administered radiostrontium unless a large dose was injected several hours before the injection of the radiostrontium, and a second dose of parathyroid extract. Consequently, the injections were made in this order in testing the effect of parathyroid extract upon the excretion of radiocalcium. The results (Table 4) demonstrate, that during the first 18 hours after the injection of labeled calcium and parathyroid extract, normal rats which had previously been injected with a large dose of parathyroid extract excreted a larger fraction of the administered radiocalcium in the urine and a smaller fraction in the feces than their controls. In the next two 24-hour periods the animals which survived the toxic effects of the parathyroid extract continued to excrete a larger amount of radiocalcium in their urine and a smaller amount in their feces than the controls. Although the calcium content of the kidneys of the experimental animals (Table 4) was not determined, it has been shown previously that multiple doses of parathyroid extract injected in normal rats produced an extreme rise in the calcium content of the kidney (Donahue, Spingarn, and Pappenheimer, 1937), and calcification in both the kidney and the heart

(McJunkin, Tweedy, and Breuhaus, 1932). From the foregoing observations it appears that parathyroid hormone in excess amount restricts the fecal excretion of calcium and promotes the urinary excretion, and that these effects are produced through the action of parathyroid hormone on the phosphorus metabolism.

It is apparent from the data presented in Table 5 that bilateral nephrectomy, like thyroparathyroidectomy, produced changes in the rat's metabolism which interfered with the excretion of exogenous calcium, and presumably with the excretion of endogenous calcium. In every case larger amounts of the administered radiocalcium were retained in the tissues of the gastrointestinal tract and in most of the other tissues of the nephrectomized rats than in the corresponding tissues of their controls. The high retention of injected radiophosphorus previously observed (Tweedy, Chilcote, and Patras, 1947) in the tissues of nephrectomized rats strongly suggests that the increased retention of radiocalcium in the tissues of our nephrectomized rats (Table 5), as well as the decreased fecal excretion of radiocalcium, was due to an increased amount of inorganic phosphorus in the tissues that immobilized a fraction of the administered radiocalcium.

It will be observed (Table 5) that the injection of parathyroid extract in the nephrectomized rats did not appear to increase the excretion of administered radiocalcium. In fact, the excretion values suggest that the injection of parathyroid extract may have retarded the excretion of the administered radiocalcium. On the other hand, when the dose of labeled calcium was increased from 0.41 to 1.75 or 2.50 mg. the nephrectomized rats, as well as their normal controls, excreted a larger per cent of the dose.

Although serum calcium determinations were not made in the case of these animals (Table 5), it has been demonstrated in previous work (Tweedy and McNamara, 1936) that serum calcium values above the normal range were seldom encountered after the injection of massive doses of parathyroid extract in nephrectomized rats. In carefully controlled experiments Stoerk (1943) appears to have prevented a fall in the serum calcium of parathyroidectomized-nephrectomized rats by the injection of parathyroid extract, but it is doubtful that an actual elevation of the serum calcium level occurred as a result of parathyroid hormone action.

SUMMARY

The urinary and fecal excretion of administered radiocalcium by rats during an 18-hour period was determined at intervals of 1 to 40 days after thyroparathyroidectomy. On an adequate diet, containing approximately 1.18% of calcium and 0.98% of phosphorus, the urinary excretion of administered radiocalcium was unchanged but the fecal excretion was

diminished and remained below the control level for several days.

Administered radiocalcium appeared to accumulate in the kidneys of thyroparathyroidectomized rats, injected with parathyroid extract, prior to the elevation of the serum calcium level, but the fractions of the injected radiocalcium excreted in the urine and feces were not increased.

Normal rats injected with toxic doses of parathyroid extract showed an increased urinary excretion of administered radiocalcium concomitant with a decreased fecal excretion.

Bilaterally nephrectomized rats excreted smaller amounts of injected radiocalcium than their normal controls. The injection of parathyroid extract in nephrectomized rats did not increase the fecal excretion of administered radiocalcium.

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THE BIOCHEMICAL EFFECTS OF SEX HORMONES IN ADRENALECTOMIZED RATS

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INTRODUCTION

A CONSIDERATION of the effects of sex hormone injections has proceeded through a study of the normal, castrated, and hypophysectomized male and female rats (Buchwald and Hudson, 1944, 1945, and 1947). Castration or hypophysectomy increased the serum acid phosphatase activity. Diethylstilbestrol decreased the serum acid phosphatase activity of the males. Diethylstilbestrol decreased the alkaline phosphatase activity of the femurs of the intact and hypophysectomized males. Testosterone propionate increased the serum alkaline phosphatase activity of the intact females. In the castrated females testosterone propionate increased the alkaline phosphatase activity of the femurs. Calcium and phosphorus of serum and feces were unchanged. To continue the study male and female rats have been adrenalectomized and injected with female and male sex hormones, respectively.

Herger and Sauer (1943), attempting to explain the delayed failures which occur in androgen control of carcinoma of the prostate, suggested that extragonadal stimuli may come from the adrenals. Huggins and Scott (1945) resorted to bilateral adrenalectomy following bilateral orchidectomy (hoping, thereby, to eliminate all sources of androgen production) in an unsuccessful attempt to control metastatic carcinoma of the prostate. Cox (1947) with unilateral adrenalectomy in two cases and bilateral in one obtained a remission of the disease but later the symptoms of metastatic carcinoma of the prostate recurred. Adrenalectomy in rats would certainly disturb their hormonal equilibrium. It was expected, therefore, that reflections of the biochemical changes in the castrated or hypophysectomized rat might be seen in the adrenalless rat.

EXPERIMENTAL

Albino rats of the Wistar strain about 130 days of age were adrenalectomized under ether anaesthesia according to the procedure outlined by Ingle and Griffith (1942). Care was taken to extirpate the gland intact and to remove completely the surrounding connective tissue. Drinking

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water was replaced with 1% NaCl. Because of the physiological effects of the absence of the adrenals the experimental procedure had to be modified from that followed for castrated and hypophysectomized rats (Buchwald and Hudson, 1945, 1947). The postoperative period before injections was shortened from 36–40 days to 4–5 days. Injections were made daily for a 28-day period.

The analytical methods were the same as those previously employed (Buchwald and Hudson, 1944).

Only the rats in which absence of both adrenals was demonstrated at autopsy comprise the adrenalectomized groups. All others are classed as subtotally adrenalectomized, whether the adrenal tissue was present because of incomplete extirpation or because of the stimulated growth and development of accessory adrenal tissue. In two rats in which there was a question of adrenal tissue present, a positive histological diagnosis was obtained.

Greep and Deane (1949) demonstrated histologically that a few cortical cells remaining after enucleation of the medulla and most of the cortex are capable of regenerating sufficient cortical tissue to provide for the rat's requirement, the secretion of cortical cells appearing about one week after enucleation.

Metabolism experiments on calcium and phosphorus similar to those reported for intact, castrated, and hypophysectomized rats seemed impractical for the adrenalectomized rat because of the uncertainty of survival. Accordingly, hope of completing the metabolism experiments was abandoned.

DISCUSSION

In all, 163 male and 153 female rats were operated for the removal of both adrenals. Of these, 24 males and 37 females survived to complete the experimental period and on autopsy proved to be free of adrenal tissue. In 12 males and 18 females or 10 per cent of the rats operated adrenal tissue was present on autopsy, either because of incomplete extirpation of the adrenal gland or development of accessory bodies of cortical tissue. Grollman (1947) points out that 7 per cent of rats have accessory bodies which lie in the connective tissue surrounding the main gland. If the gland is injured on extirpation or if any accessory tissue is left the remaining cortical cells may proliferate and replace the cortical tissue. As a result of our observations on the adrenalectomized rat the increase in serum acid phosphatase activity affords a criterion of the absence of cortical adrenal tissue.

Injection of diethylstilbestrol in doses of 0.1 mg. per day produces a toxic reaction in the adrenalectomized male rat. Absence of the adrenals

causes a loss of appetite which together with the effect of diethylstilbestrol the male rats are unable to survive for more than 7–10 days.

Reduction of the dose of diethylstilbestrol or its substitution with the natural hormone, theelin,[1] was still too toxic to be tolerated by the adrenalless rats. Because of the failure of the adrenalectomized male rat to survive an effective period of estrogen injection we are unable at present to present a full set of data for this group. Attempts to complete this part of our

TABLE 1. EFFECTS OF INJECTING OIL INTO ADRENALECTOMIZED MALE RATS

	1 Noninjected	2 Oil	3*
Number of rats	12	12	
Weight difference, gm.	3 ±6.09	−4 ±3.85	0.97
Femurs, fresh weights, gm.			
1.	0.78 ±0.018	0.80 ±0.017	0.81
2.	0.78 ±0.018	0.80 ±0.016	0.83
Femur, weight of ash, mg.	341.3 ±7.87	361.3 ±5.82	2.01
Sex organs, weight, gm.	2.56 ±0.034	2.63 ±0.055	1.08
Serum			
Calcium, mg. %	9.95 ±0.140	9.84 ±0.098[1]	0.64
Phosphorus, mg. %	8.15 ±0.209	7.99 ±0.174[1]	0.59
Phosphatase, units per 100 cc.			
Acid	13.2 ±0.34	14.6 ±0.44	2.52
Alkaline	93.6 ±4.58	82.8 ±4.93	1.60
Bone			
Alkaline phosphatase, units per gm.	16.4 ±1.04	17.0 ±1.07	0.40

[1] 10 rats.
* Comparison between 1 and 2.

experiment will be continued, improving the regime for the adrenalectomized rats by use of a low potassium diet from the weaning age of the rats through the experimental period.

Tables 1 and 2 present the mean results with their probable errors. The comparison is shown statistically in Column 5 of Table 1 and Columns 4 and 5 of Table 2. These values had to be over three to be considered significant.

Table 1 presents the data for the adrenalectomized male rats noninjected and oil injected (0.1 cc. inventol per day for 28 days.) As in the normal, castrated, and hypophysectomized rats there is no difference between the noninjected and the oil injected groups.

In Table 2 are presented the data for the adrenalectomized female rats, noninjected, oil injected, and testosterone propionate injected animals. There is an indication of a decrease in weight of ash of the femurs in

[1] Theelin in oil, Parke-Davis & Co.

the oil injected group as compared with the noninjected group. However, it must be observed that there is a large variation within the oil injected group. There is no other significant difference between these two groups of adrenalectomized female rats. Similarly, little variation occurred between these two groups in the normal, castrated, and hypophysectomized female rats as previously reported. In the testosterone propionate injected rats there is an increase of body weight over the controls. The weight of ash of the femurs of the testosterone propionate injected rats shows a little

TABLE 2. EFFECTS OF INJECTING TESTOSTERONE PROPIONATE INTO ADRENALIZED FEMALE RATS

	1 Noninjected	2 Oil 0.1 cc./da. for 28 da.	3 Testosterone propionate 0.2 mg./da. for 28 da.	4*	5**
Number of rats	12	13	12		
Weight difference, gm.	−1 ±2.74	5 ±1.44	29 ±3.77	1.94	5.95
Femurs, fresh weights, gm.					
1.	0.60±0.007	0.58±0.009	0.62±0.030	1.75	1.28
2.	0.60±0.007	0.58±0.010	0.62±0.005	1.64	3.58
Femur, weight of ash, mg.	279.3 ±2.80	258.4 ±4.66	283.1 ±2.82	3.84	4.54
Sex organs, weight, gm.	0.37±0.032	0.32±0.030	0.66±0.002	1.14	9.14
Serum					
Calcium, mg. %	9.61±0.234[3]	10.05±0.208[3]	9.88±0.101[2]	1.41	0.74
Phosphorus, mg. %	7.90±0.611[2]	7.45±0.288[3]	7.48±0.299[2]	0.67	0.07
Phosphatase, units per 100 cc.					
Acid	16.5 ±0.86	19.4 ±0.72	14.3 ±0.64	2.59	5.29
Alkaline	86.3±5.06	93.9 ±2.94	86.4 ±4.89	1.30	1.31
Bone					
Alkaline phosphatase, units per gm.	12.0 ±0.780	12.5 ±0.58	12.1 ±0.92	0.51	0.37

* —Comparison between 1 and 2.
** —Comparison between 2 and 3.
[2] 7 rats.
[3] 5 rats.

increase over the oil injected but none over the noninjected. As in the castrated and hypophysectomized rats, testosterone propionate produced an increase in the weight of the sex organs. In the normal rat, however, an atrophy occurred.

The acid phosphatase activity of the serum of the testosterone propionate injected rats gives an average value which is less than either the noninjected or oil injected. The difference from the oil injected group is the greatest. This difference was not observed in the normal, castrated, or hypophysectomized rats.

Comparisons can be made between Tables 1 and 2 here presented and in the report for the intact rat (Buchwald and Hudson, 1944). Removal of the adrenals reduced the weight of the male and female rats compared to the intact rats. This weight reduction is further reflected in reduction in the fresh weight of the femurs as well as the ash of the femurs. Adrenalectomy in the female rats produced a definite decrease in the sex organs but did not affect the testicles of the males. The average serum calcium of the

adrenalectomized rats is lower than the intact. The difference is significant in the male oil injected and the male and female noninjected rats.

The average serum phosphorus of the adrenalectomized rat is definitely increased in comparison with the intact. The difference is significant in the male noninjected and male and female injected rats. The increase in serum phosphorus may be due to a deranged kidney function which is an accepted result of adrenal insufficiency. The decrease in calcium could be a compensating mechanism for the increase in the serum phosphorus.

The most spectacular observation of the result of adrenalectomy in both male and female rats is the marked increase in serum acid phosphatase activity. Reference has been made above to the marked increase in serum acid phosphatase activity as a suggested criterion for the completeness of ablation of the adrenal gland in rats. In female rats either noninjected or oil injected as well as those receiving testosterone propionate the serum acid phosphatase activity progressively increased from the normal through the castrated, hypophysectomized, and adrenalectomized rats. In males noninjected or oil injected the serum acid phosphatase activity showed an increase in the castrated rat and a further increase in the hypophysectomized. In the adrenalless rat the serum acid phosphatase activity is greater than the castrated but less than the hypophysectomized.

The data presented seems to be proof that in both male and female rats the level of serum acid phosphatase activity is controlled by the adrenal and testicle. The serum acid phosphatase activity, though increased over the normal and castrated, was not as high as in the hypophysectomized male in which the testes and adrenals were atrophied. In the female removal of the adrenals produced an animal in which the serum acid phosphatase activity level was higher (16.5 units/100 cc. noninjected and 19.4 units/100 cc. oil injected), having removed the source of androgenic hormone. Administration of the androgen in the form of testosterone propionate reduced the level of serum acid phosphatase activity (14.3 units/100 cc.) to the level found in the adrenalless male (13.2 units/100 cc., noninjected, and 14.6 units/100 cc., oil injected).

The average serum alkaline phosphatase activity is increased in all groups by adrenalectomy, significantly so in all but the male oil injected and female testosterone injected rats. However, in all groups the serum alkaline phosphatase activity is highest in the hypophysectomized rats.

The bone alkaline phosphatase activity like the serum alkaline phosphatase activity is increased in every group by adrenalectomy. The increases are significant in every group except the testosterone propionate injected.

Adrenalectomy produced an organism in which the serum acid and alkaline phosphatase and the bone alkaline phosphatase activities as well as the serum phosphorus were all at a higher level than in the intact animal.

A slight increase in the alkaline phosphatase activity of the liver and a slight decrease in kidney following adrenalectomy were found by Vail and Kochakian (1947) using histochemical technique.

The acid phosphatase activity of the prostate gland of the human and of the rat is under investigation by the authors as a separate study. Data so far accumulated suggests that adrenalectomy causes an increase in the acid phosphatase activity of the rat prostate gland.

Neither hypophysectomy nor adrenalectomy, even if either were practical as therapeutic measures, are indicated by our results in rats for the treatment of metastatic carcinoma of the prostate. Although adrenalectomy produces in every group a high bone alkaline phosphatase activity which is recognized as a favorable condition for the healing of bony lesions, the serum acid phosphatase activity is high. In the human a high serum acid phosphatase activity is an unfavorable prognosis in carcinoma of prostate metastatic to bone. Hypophysectomy in rats even produced a lowering of the bone alkaline phosphatase activity as well as an increase of serum acid phosphatase activity, both unfavorable conditions for the healing of bony lesions metastatic from carcinoma of the prostate.

SUMMARY

Daily subcutaneous injections of 0.2 mg. of testosterone propionate were given adrenalectomized female rats for a 28-day period. In these rats no changes were produced in the serum calcium and phosphorus and the alkaline phosphatase activity of the serum and bone following testosterone propionate injections. A decrease in the serum acid phosphatase activity occurred.

No data was obtained for the injection of diethylstilbestrol because of its toxicity for the adrenalectomized male rat.

Adrenalectomy produced an increase in the serum acid and alkaline phosphatase and bone alkaline phosphatase activities in both male and female rats.

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THE LOCAL ACTION OF ADRENOCORTICAL STEROIDS ON EPIDERMIS AND CONNECTIVE TISSUE OF THE SKIN[1]

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HYPERACTIVITY of the adrenal cortex induced by treatment of rats with ACTH results in a general suppression of growth. In the skin, the epidermis becomes thinner, hair stops growing and the dermal connective tissue assumes a more compact arrangement (Baker, Ingle, Li and Evans, 1948). These effects appear to be mediated by C-11 oxygenated steroid which are secreted by the adrenal glands. If one of these compounds, cortisone, or an extract of the adrenal cortex is applied directly to the skin, similar changes are induced locally in the epidermis and hair follicles (Baker and Whitaker, 1948). In these early short-term experiments consistent changes in the connective tissue were not observed but, subsequently, it was demonstrated that adrenocortical extract inhibits growth of granulation tissue by local action (Baker and Whitaker, in press).

This report deals with the cutaneous modifications elicited by prolonged local treatment of non-traumatized skin with adrenocortical compounds. The experiments had two general purposes: (1) to observe the changes occurring in the epidermal cells and fibro-elastic connective tissue of the dermis and (2) to find out if a refractory state will develop toward the local action of adrenocortical steroids in rats treated for long periods of time.

MATERIALS AND METHODS

Fifty-nine adult rats of the Long-Evans strain and 7 of the Wistar strain were used. Various adrenocortical hormones in alcoholic solution were applied daily to the skin of the neck caudal to the right ear of 43 of these animals. Twenty-three received the solvent, 25% alcohol, in a similar manner. The duration of treatment is given in tables 1 and 2. In addition to the animals represented in these tables, some were treated with extract for as long as 180 days. Eleven-dehydro-17-hydroxycorticosterone (cortisone) and 17-hydroxycorticosterone (compound F)[3] were used in daily doses of 25 to 100 μg. dissolved

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in 0.1 cc of 25% alcohol. The extract was derived from hog adrenal glands and was made up in 25% alcohol in a concentration which in terms of liver glycogen units was equivalent to 1 mg. of cortisone per cc. Accordingly, since 0.1 cc. of the extract was administered daily, the doses of extract used were equivalent to the highest doses of cortisone when compared by the liver glycogen test. Control rats were treated similarly with 25% alcohol. At various periods after the initiation of treatment, biopsies of the skin were taken from symmetrical areas behind the ears for microscopic examination. Samples were fixed in Carnoy's or Bouin's fluid and stained with eosin and methylene blue (citric acid-disodium phosphate buffer, pH 4.3 to 5.8), iron hematoxylin and Masson, hematoxylin and eosin, the Hotchkiss (1948) periodic acid-leucofuchsin technique and Weigert's stain for elastic fibers.

Epidermal cell area was ascertained on sections which were fixed in Carnoy's fluid and stained with eosin and methylene blue, this area being considered as an index of cell volume. The cells measured were located in the stratum germinativum. The width and height of the cell were measured with an ocular micrometer and since most cells approached an elliptical shape, their area was calculated by the formula: area $=\pi ab/4$, with a and b representing the longest and shortest axes. Cell measurements were made according to the following procedure. Six adjacent cells were measured in each of three different locations on a single section. The locations chosen were the two ends and the middle, and in order to decrease the personal factor, the specific area was selected under low magnification before measurement with an oil immersion objective. Thus, cell size was computed from the average of the measurements of 18 cells.

The thickness of the dermis was determined with an ocular micrometer. Measurements were made from one end of a section to the other at intervals of about 300 μ and included the connective tissue only to the point where it met either the panniculus adiposus or the panniculus carnosus muscle.

The statistical work was done by the staff of the Statistical Research Laboratory of the University of Michigan. The Student Fisher t formula was used throughout.

OBSERVATIONS

All of the cutaneous modifications to be described were limited to the area to which adrenocortical hormones had been applied. Grossly, after several weeks of treatment the skin on the right, treated, side of the neck assumed a blanched appearance. Folds formed by lifting the skin between the fingers flattened out more slowly than on the left side of the neck.

Epidermis. In the area of skin to which adrenocortical hormones were applied, most of the rats showed thinning of the epidermis which was accompanied by a moderate reduction in the number of cell layers composing the stratum germinativum and stratum granulosum. The epidermal cell area was reduced significantly on the right, treated, side of the neck in male rats which received daily doses of 100 μg. of cortisone or its equivalent in hog adrenocortical extract for an average of 91 days (Table 1). The magnitude of the effect was related directly to the duration of treatment. A decrease in size of these cells was not observed in other animals which were treated with lower doses. The degree of hormonal effect was not modified by the age or weight of the experimental rats and the solvent,

25% alcohol, induced no change in cell size. Basophilia was retained by the epidermal cells in the area receiving the adrenal hormones.

On the other hand, in female rats treated with 100 μg. of cortisone or its equivalent in extract, a statistically insignificant reduction in epidermal cell area was obtained (Table 1). Equally inconclusive results were obtained on 13 female rats not represented in table 1 which were treated variously with daily doses of 100 μg. of desoxycorticosterone, 25 μg. of cortisone, 17-hydroxycorticosterone or the equivalent dosage in adrenocortical extract.

TABLE 1. EFFECT OF ADRENOCORTICAL STEROIDS AND ALCOHOL ON EPIDERMAL CELL AREA

Sex	No. of rats	Treatment	Mean days of treatment	Mean area (μ^2)		"t"
				Left	Right	
Male	7	ACE; E[1]	91 ± 28.4[2]	42.9 ± 3.0	30.1 ± 5.3	5.58 P < .01[3]
Male	4	Alc.	77 ± 34.3	43.6 ± 6.2	41.3 ± 4.8	0.59 P > .50
Female	8	ACE; E[1]	81 ± 23.4	38.6 ± 4.5	36.4 ± 5.5	0.88 P > .10
Female	6	Alc.	84 ± 11.6	37.8 ± 3.0	38.2 ± 3.0	0.20 P > .50

[1] ACE-Hog adrenal extract; E = 11-dehydro-17-hydroxycorticosterone. 100 μg. or its equivalent per day.
[2] Standard deviation.
[3] A p value < .05 is considered significant.

Dermis. Measurement of dermal thickness on the right and left sides of the neck in 41 animals treated with extract, cortisone or compound F revealed that these steroids caused this layer of the integument to become thinner in both sexes (Table 2). However, this difference was statistically significant in the case of only the females. It is believed that within this series the shorter mean period of treatment to which the males were subjected, accounted for their failure to show a significant effect because all male rats which were treated for 60 days or longer possessed a thinner dermis in the treated area. Thus, figures 1 and 2 demonstrate that a great reduction in thickness of the dermis occurred in males when the duration of the experiment was extended to 112 days. In both sexes treatment with the vehicle, 25% alcohol, failed to produce a significant thinning of the dermis (Table 2).

In the normal rat, areas of active hair growth spread in waves over the body so that portions of skin containing quiescent or active hair follicles exist adjacent to each other. The dermis of these animals is somewhat more compact in regions where hair is not growing. Therefore, it was necessary to determine the extent to which local thinning of the dermis caused by adrenocortical steroids was an indirect outcome of the cessation in growth

of hair. Accordingly, measurements of the dermis on the left, non-treated, side of the neck in a series of rats were grouped according to the presence or absence of growing hair in that region and a comparison was made of the means. Table 3 shows that although the dermis in areas of inactive hair growth in both sexes was slightly thinner than that of active areas,

TABLE 2. THE EFFECT OF ADRENOCORTICAL STEROIDS ON THICKNESS OF THE DERMIS

Sex	No. of rats	Treatment	Mean days of treatment	Mean thickness (μ)		"t"
				Left	Right	
Male	19	ACE; E; F[1]	49 ± 37[2]	554 ± 150.2	467 ± 167.1	1.68 P > .10[3]
Male	4	Alc.	57 ± 31	400 ± 87.6	390 ± 87.6	0.09 P > .09
Female	22	ACE; E	57 ± 26	351 ± 76.4	280 ± 69.0	3.20 P < .01
Female	3	Alc.	87 ± 10	288 ± 38.4	277 ± 52.2	0.28 P > .08

[1] ACE = Hog adrenal extract; E = 11-dehydro-17-hydroxycorticosterone; F = 17-hydroxycorticosterone. The doses were 25 or 100 μg. of compounds E and F per day and the equivalent in extract.
[2] Standard deviation.
[3] A p value < .05 is considered significant.

TABLE 3. THE EFFECT OF SEX AND STATE OF HAIR GROWTH ON THICKNESS OF THE DERMIS ON THE LEFT, NON-TREATED SIDE

Sex	No. with no hair growth	Mean thickness (microns)	No. with hair growth	Mean thickness (microns)	"t"
Males	11	511.1 ± 196[1]	12	542.1 ± 130.3	0.45 P > .50[2]
Females	14	335.1 ± 73	11	353.4 ± 80.2	0.60 P > .50

[1] Standard deviation.
[2] A p value < .05 is considered significant.

the difference was not statistically significant. Therefore, the adrenocortical hormones induced a thinning of the dermis by some other process in addition to the inhibition in growth of hair.

When treatment with adrenal extract was extended to 112 days significant histological changes were observed in the dermis. In some cases the collagenous fibers lost their sharp boundaries and appeared to be fused together giving the dermis a more homogeneous appearance (Figures 1 and 2). However, in other instances, the outline of the collagenous fibers was quite distinct even though the dermis as a whole was much thinner. Preparations stained with the Weigert technique showed that elastic fibers had not been reduced by the adrenal hormones. In fact, they were

more prominent and concentrated on the right than on the left side of the neck (Figures 3 and 4). Thus, it is evident that the reduction in thickness of the dermis was due primarily to loss of substance from the collagenous fibers. In sections of the skin treated with Hotchkiss' periodic acid-leucofuchsin procedure, the basement membranes beneath the epidermis, surrounding the capillaries, sebaceous glands and hair follicles, and the stroma between the contractile cells of the arrectores pilorum muscle stained with an intensity on the right side which was equal to or greater than that of the left side (Figures 6 and 7). It is to be inferred that in these locations the connective tissue contains substances of high molecular weight such as a glycoprotein (Hotchkiss, 1948) and that the adrenal hormones did not cause their removal.

The number of cells in the dermis was reduced by the extract and those cells (such as fibroblasts) which remained, possessed shrunken nuclei. These changes in the cells were most pronounced in the superficial region of the dermis. Sebaceous glands were smaller. The panniculus adiposus was thinner than that of the control side or was completely absent (Figure 2).

Refractoriness. In 2 male rats, treatment of the right side of the neck

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EXPLANATION OF FIGURES

FIG. 1. Skin from the left, non-treated side of the neck showing a thick epidermis, large sebaceous glands and a growing hair follicle. The dermis and subcutaneous fat layer are thick. Carnoy fixation; hematoxylin and eosin. ×57.

FIG. 2. Skin from the right, treated side of the neck of the same rat as that illustrated in figure 1. Adrenal extract was applied to this area for 112 days, the daily dose being equivalent to 100 μg. of cortisone. The epidermis is thin, sebaceous glands smaller and the hair follicles are inactive. The dermis is thinner, the collagenous fibers appearing to be fused into a rather homogeneous mass. Subcutaneous fat is removed. Technique as in figure 1.

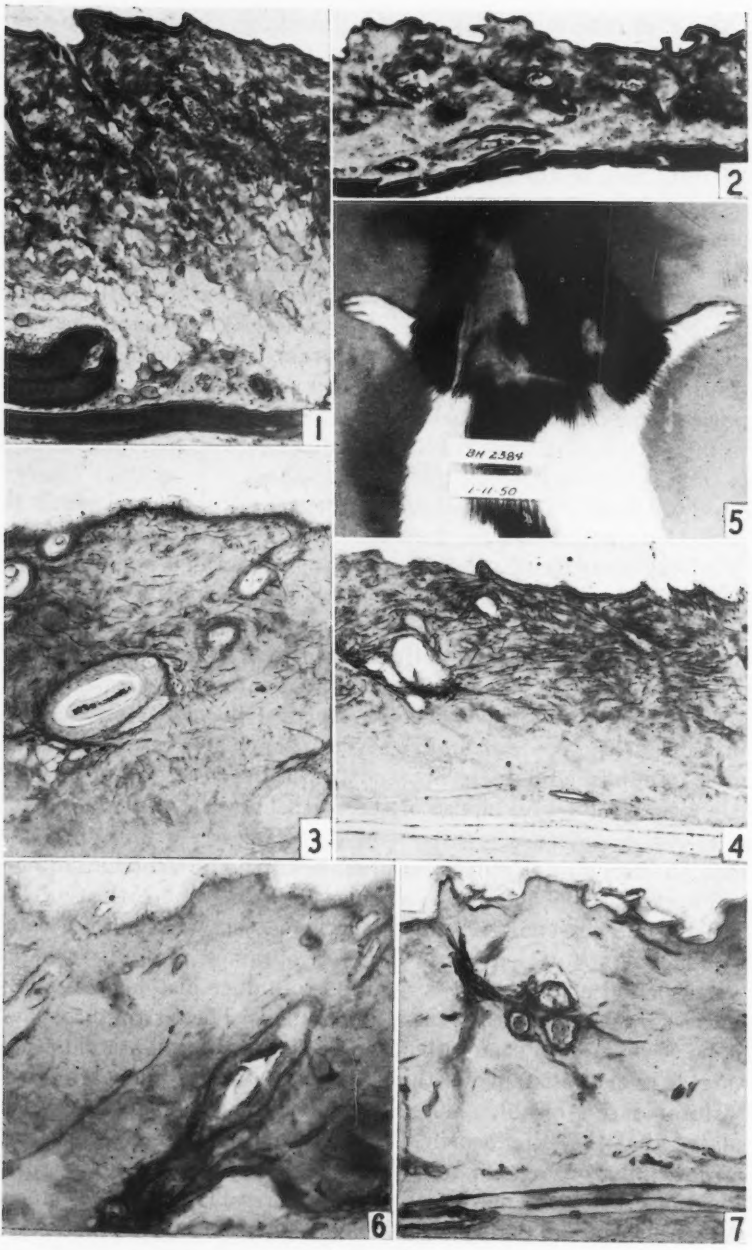
FIG. 3. Skin from the left side of the neck showing the sparse distribution of elastic fibers and their greater concentration around hair follicles and sebaceous glands. Carnoy fixation; Weigert's resorcin-fuchsin. ×96.

FIG. 4. Skin from the right side of the neck of the same rat as that illustrated in figure 3, treated in this area with adrenal extract for 81 days. The concentration of elastic fibers is increased. Technique as in figure 3.

FIG. 5. This rat was treated daily on the right side of the neck with adrenal extract for 180 days in doses equivalent to 100 μg. of cortisone. Growth of hair was suppressed for about 160 days. This illustrates the resumption in growth of hair which occurred during the last week of treatment.

FIG. 6. Left side of the neck of the same animal as that illustrated in figure 3, showing basement membranes beneath the epidermis and around hair follicles, sebaceous glands and capillaries. Carnoy fixation, Hotchkiss' periodic acid-leucofuchsin. ×128.

FIG. 7. Right side of the neck of the animal illustrated in figure 4. Basement membranes are stained as well as in figure 6. An arrectores pilorum muscle is at the left of the hair follicles. Technique as in figure 6.



FIGS. 1-7

with 0.1 cc. of extract per day was continued for 180 days. Two animals received 25% alcohol. This is the longest period of time any of our rats have been treated with adrenal hormones by local application. In the extract-treated rats complete inhibition in growth of hair was maintained for about 160 days but during the last 2 weeks, growth of hair was resumed on the right side of the neck in spite of continuation of the treatment. Microscopic examination of the skin showed that recovery had been rather complete. The epidermis in 1 rat was still somewhat thinner than that of the left side. Hair follicles and sebaceous glands were normal, the former being in a state of active growth. The dermis was only slightly thinner in the treated area. The panniculus adiposus was thicker than on the left side of the neck of the same rat or in the control rats. These findings show clearly that in time the skin becomes refractory to the local action of the adrenal steroids contained in this extract.

DISCUSSION

It has been demonstrated that adrenocortical steroids, particularly those of the C-11 oxygenated type, may cause regressive changes in all of the epithelial constituents of rat skin including epidermis, hair follicles and sebaceous glands. There are 3 possible explanations for the thinning of the epidermis: an increased rate of cornification, reduction in the rate of cell division or reduction in size of the constitutent cells. No evidence was obtained which would suggest an accelerated rate of keratinization. Preliminary studies of the effect of adrenocortical hormones on the rate of cell division in the epidermis have given inconclusive results (Baker and Schairer, unpublished data). This study did show that at least in the case of male rats, a reduction in size of the epithelial cells contributed to the thinning of the epidermis.

These findings do not appear to provide a rational explanation of why adrenal hormones alleviate the symptoms of rheumatoid arthritis (Hench, *et al.* 1949) and disseminated lupus erythematosus (Grace and Combes, 1949). The therapeutic effects in man are obtained promptly whereas a prolonged period of treatment is required to induce histological modification of the cutaneous connective tissue in animals. These structural changes might be secondary to some other action of the adrenal steroids such as an increase in the resistance of the ground substance to depolymerization by hyaluronidase (Opsahl, 1949). Dougherty and Dougherty (1950) have postulated that the adrenocortical hormones act in these diseases chiefly as anti-inflammatory agents and, therefore, it is pertinent that structural modifications may be induced in apparently normal fibro-elastic connective tissue in which inflammatory reactions are not involved.

The development in the skin of a state of refractoriness to the action

of the adrenal hormones presents the possibility of loss in effectiveness of these hormones when administered to patients for long periods of time. In addition to this factor, loss in the effectiveness of extended treatment with heterologous ACTH may be expected due to the formation of anti-hormones which have been demonstrated to appear in animals treated with ACTH (Chase, 1949; Gordon, 1949). Finally, this recovery demonstrates that the cutaneous modifications induced by the local action of adrenocortical hormones, do not represent permanent damage.

SUMMARY

The prolonged percutaneous application of adrenocortical hormones modified the histology of the skin, the changes induced being limited to the area of treatment. The epidermis became thinner and, in males, the size of the epidermal cells was reduced. Growth of hair ceased and sebaceous glands became smaller. The thickness of the dermis was reduced, apparently due to loss of substance from the collagenous fibers, the elastic fibers remaining numerous in spite of the treatment. Fibroblasts and other cells of the dermal connective tissue were fewer in number.

The development of a state of refractoriness to the action of the hormones was demonstrated by the resumption in growth of hair in the area of application when treatment was continued for 180 days.

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A STUDY OF THE ABSORPTION CHARACTERISTICS OF IODINE-131 LABELED IODINATED CASEIN IN SOME MAMMALS[1]

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INTRODUCTION

INVESTIGATION on the formation and thyroidal potency of synthetic iodinated proteins dates back to the latter part of the nineteenth century, following Baumann's discovery of iodine in organic combination in the thyroid gland in 1895.

Recently, Reineke and Turner (1943) have reported improved techniques for the synthesis of iodocasein which shows a high degree of thyroidal potency and have carried out investigations to determine if this material influences various productive processes in domestic animals. Comparisons have been made of the amounts of iodocasein required to produce a lactation response in dairy cattle when administered orally or injected subcutaneously. The results indicated that iodocasein is utilized only slightly when given orally. In sheep, only 5 per cent as much iodocasein was required by subcutaneous injection to bring about a standard weight loss as when given orally. When the iodocasein was placed directly in the abosmasum through a permanent cannula in order to bypass the rumen, no increase in utilization was observed (Reineke and Turner, 1942).

It thus appeared that the rumen was not the site of inactivation of the active principle. Oral utilization was increased slightly by the preliminary hydrolysis of the iodocasein with acid, indicating that poor utilization obtained with the whole protein may be partly due to incomplete digestion.

It was felt that further light could be thrown on the subject of absorption of iodocasein from the alimentary tract of ruminants if, prior to administration, it was labeled with radioactive iodine-131. By doing so, any absorption may be noted quite simply by carrying out assays of the blood for radioactivity at periodic intervals.

The use of labeled iodocasein also facilitates the identification of some

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of the metabolites appearing in the blood. Although these substances are present in amounts too small to be identified by gravimetric methods, the use of the carrier technique somewhat simplifies this problem.

Therefore, iodocasein labeled with iodine-131 was prepared and administered to rats and sheep. The labeled metabolites appearing in the plasma of the rat and the sheep and the milk of lactating ewes were studied. Particular emphasis was placed on the determination of the distribution of iodine-131 between the thyroxine and diiodotyrosine fractions, as these substances are the ones holding the interest of workers attempting to settle the controversial question of the nature of the circulating thyroid hormone (Thompson *et al.* 1929; Harrington, 1935–44; Salter, 1940; Means *et al.* 1941; Williams *et al.* 1947; Taurog *et al.* 1949).

EXPERIMENTAL

Iodocasein labeled with iodine-131[3] was prepared by the method of Frieden, Lipsett and Winzler (1948), a modification of the method of synthesis of Reineke and Turner (1943); twenty millicuries of iodine-131 were added to a methanol solution of powdered iodine-127 which was used as carrier. An iodocasein of higher specific activity may be made quite simply by increasing the amount of iodine-131 added per gram of carrier iodine-127.

The radioactive assay of the labeled iodocasein was carried out in the following manner: The iodocasein solution was counted by pipetting 0.5 ml. of the solution on a filter strip, $2\frac{1}{2}''$ by $2''$ which was then dried with infra-red light. This in turn was placed on a piece of backing paper, $3''$ by $2\frac{1}{2}''$ made from mimeograph paper. The filter strip was sealed to the backing paper with a pane of Dupont cellophane, $3''$ by $2\frac{1}{2}''$ set down with scotch tape. The mounted filter strips were fastened around an Eck and Krebs type counting tube and excellent reproduction of geometry was obtained. This method was also used for the determination of the amount of radioactivity present in the blood and milk samples. In the identification of thyroxine in rat and sheep plasma by the carrier technique, an end-window tube was used. All counts determined in this work were corrected for decay, background and coincidence when necessary.

When iodocasein was prepared using iodine-127, the final product gave a negative test for inorganic iodine using acid potassium permanganate as the testing agent. Similar results were obtained by Reineke and Turner (1943).

It was also found that when iodine-131 labeled iodocasein was given orally by stomach tube to large white rats in the post-absorptive state, no

[3] The iodine-131 used in this research was obtained on allocation by the U. S. Atomic Energy Commission.

radioactivity was detected in the thyroids during the first 24 hours, although there was a considerable amount present in the circulating blood. As the capacity of the thyroid to rapidly remove inorganic iodine-131 from the bloodstream is well-known (Hertz *et al.* 1938; Perlman *et al.* 1941; Leblond, 1942; Findley, 1947), this would indicate that there is no inorganic iodine associated with the iodocasein or formed during the absorption of the iodocasein from the alimentary tract of the rat.

The distribution of iodine-131 in rat plasma following oral administration of labeled iodocasein was carried out prior to using larger more expensive domestic animals in order to establish the best possible techniques.

Ten milliliters of labeled iodocasein, counting 2,000,000 c.p.m./ml. and containing 0.027 g./ml. of iodocasein was given orally to 4 large rats. The blood was removed 10 hours later, as at this time the blood level of

TABLE 1. THE DISTRIBUTION OF IODINE-131 IN RAT PLASMA

Nature of material	Total activity	Activity expressed as % of plasma activity
5 ml. of plasma	8160 c.p.m.	100%
Butyl alcohol extract of plasma	3520 c.p.m.	43%
Thyroxine fraction of plasma	500 c.p.m.	6%
Diiodotyrosine-inorganic iodine fraction of plasma	3010 c.p.m.	36%

radioactivity is at its peak. The plasma was obtained by centrifugation and was then shaken up with butyl alcohol which is reported to extract thyroxine, diiodotyrosine and inorganic iodine from plasma (Trevarrow, 1929; Elmer, 1934; Wilmans, 1943; Leblond, 1946, and Leland, 1946). This was further separated into 2 thyroxine fractions and a diiodotyrosine-inorganic iodine fraction using Blau's reagent (Blau, 1933).

The pattern of distribution of iodine-131 in rat plasma is shown in table 1.

About 60 per cent of the iodine-131 in the rat plasma is not extractable with butyl alcohol under the conditions of extraction described. The composition of this residue is a matter for conjecture, possibly consisting of other iodinated amino acids not soluble in butyl alcohol.

Thyroxine was positively identified employing the carrier technique. Fifty milligrams of recrystallized thyroxine was added as carrier to the butyl alcohol extract of the plasma which was then concentrated to dryness. The thyroxine was recrystallized from potassium carbonate solution twice, followed by recrystallization from 70 per cent alkaline ethanol and dilute sodium hydroxide. A constant specific activity of 32 c.p.m./mg. was attained after each recrystallization, indicating that the radioactivity present in the butyl alcohol-thyroxine fraction was the same substance as the added carrier, namely thyroxine.

Next, labeled iodocasein was administered intravenously to sheep. The sheep used were 1½ year old castrated males, predominantly of Rambouillet breeding weighing approximately 130 lb. and having an estimated blood volume of 2500–3000 ml.

Thirty milliliters of iodocasein counting 920,000 c.p.m./ml. and containing 0.032 g./ml. of iodocasein was injected into the jugular veins of two sheep. Two other sheep were given 50 ml. by the same route. Blood samples were taken for radioactive assay from the vein at appropriate intervals.

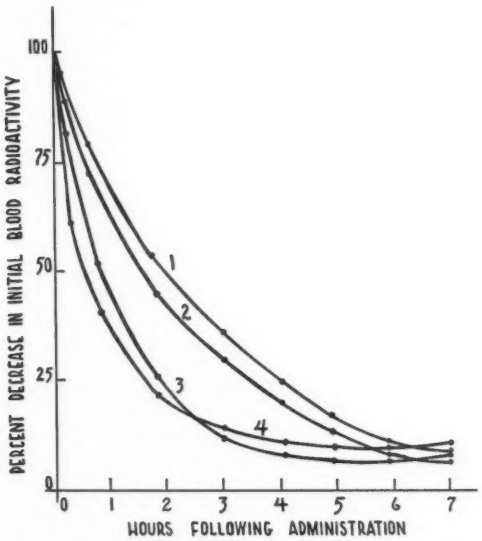

FIG. 1. Decrease in blood radioactivity expressed as a percentage of the initial activity present. The sheep represented by curves 1 and 2 received 30 ml. of radioactive iodocasein intravenously. Sheep 3 and 4 received 50 ml. of radioactive iodocasein intravenously.

As shown in Figure 1, the decrease in activity is expressed as a per cent of the activity of the first blood sample drawn immediately after administration. From this it is seen that 90 per cent of the activity of iodocasein is removed from the bloodstream in 7 hours and that the larger dose of iodocasein shows a per cent decrease in activity per hour that is proportionally higher than that shown for the smaller dose.

Following this, the absorption of labeled iodocasein from the alimentary tract of the sheep was studied. Four routes were taken, oral, ventral sac of the rumen, small intestine and cecum. The sheep used were the same age, weight and breed as those mentioned above. Permanent fistulas leading into the ventral sac of the rumen, small intestine and cecum were sur-

gically established 9 months after birth.[4] Oral administration was achieved by passing a rubber catheter through the nostril and into the esophagus. The material was introduced into both the small intestine and cecum by means of a rubber catheter passed through the fistulas leading into these organs. A 12″ metal catheter inserted through a fistual in the rumen wall was used to deposit the material in the ventral sac of the rumen. The animals received their usual diet of alfalfa hay and shelled corn during the

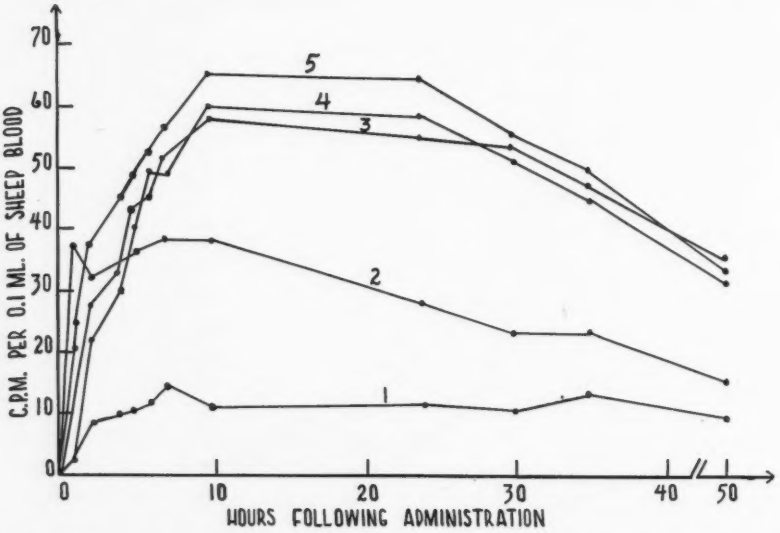

FIG. 2. The effect of route of administration of radioactive iodocasein on blood radioactivity. 1.—75 ml. of radioactive iodocasein in the cecum. 2.—75 ml. in the small intestine. 3.—75 ml. in the ventral rumen sac. 4.—75 ml. orally. 5.—200 ml. orally.

experiments. Feed was not withheld at any time, since it was impractical to attempt to treat ruminants in the post-absorptive state.

Each of four sheep was given 75 ml. of iodocasein containing 0.032 g./ml. of iodocasein counting 1,400,000 c.p.m./ml. by the 4 routes of administration formerly outlined. A fifth sheep was given 200 ml. of material orally to study the effect of larger dosage on the level of absorption. Blood samples were removed at appropriate time intervals over a 50 hour period and assayed for radioactivity using the filter strip method. As shown in Figure 2, the highest blood levels were reached following oral administra-

[4] The authors are indebted to Dr. J. F. Bullard for the surgical establishment of the various fistulae.

tion, rather than from small intestine administration as might be expected if the material were inactivated in the rumen. Secondly, although the 200 ml. dose was four times as large as the smaller dose, the blood level was not significantly higher following its ingestion. Thirdly, there was little absorption from the cecum.

The distribution study of iodine-131 in sheep plasma was carried out in the same fashion that was employed for rat plasma. Sheep weighing approximately 100 lb. each were given 100 ml. of iodocasein counting 5,500,000 c.p.m./ml. containing 0.032 g./ml. of iodocasein orally via stom-

TABLE 2. THE DISTRIBUTION OF IODINE-131 IN SHEEP PLASMA

Nature of material	Total activity	Activity expressed as % of plasma activity
5 ml. of plasma	22,270 c.p.m.	100%
Butyl alcohol extract of plasma	12,000 c.p.m.	54%
Thyroxine fraction of plasma	3,900 c.p.m.	10%
Diiodotyrosine-inorganic iodine fraction of plasma	8,101 c.p.m.	39%

ach tube. Ten hours later, 50 ml. of blood was removed from the jugular vein of each sheep and the plasma obtained by centrifugation. The plasma from the sheep was pooled and a 5 ml. sample taken for study.

The method of Taurog and Chaikoff (1949) was used to obtain the fractions studied and the results are shown in Table 2.

From these figures it will be seen that, as in the rat, only a little more than 50 per cent of the radioactivity of the plasma was extractable with butyl alcohol. As before, thyroxine was identified employing the carrier technique, giving a constant specific activity of 9.8 c.p.m./mg. of thyroxine following each recrystallization.

The effect of iodocasein in increasing the milk production of domestic animals is well known (Graham, 1943; Turner, 1940; Reineke *et al.*, 1942; Blaxter, 1945; and Janvier, 1948). In connection with another experiment begun in the Spring of 1949, Andrews *et al.* (1949), 3-year-old lactating ewes of Hampshire and Rambouillet breeding were fed iodocasein in an attempt to stimulate milk production and thereby increase the growth rate of the lambs. Six to eight weeks after the feeding experiment was begun, the group of lambs being suckled by the ewes fed iodocasein had on the average a significantly lower weight than the lambs being suckled by ewes which had not been fed iodocasein. The mortality rate of the control lambs was lower. However, no adverse effects were noted in the ewes themselves following the feeding of iodocasein.

It was thought that the iodocasein ingested by the ewes might possibly

be excreted in the milk and be adversely affecting the lambs. Reineke and Turner (1943) claim that no iodocasein passes into the milk of cows following administration.

To check this hypothesis, six lactating ewes were each given 150 ml. of labeled iodocasein orally by stomach tube. The material counted 544,000 c.p.m./ml. and contained 0.02 g./ml. of iodocasein. Milk and blood samples were taken over a 3-day period for radioactive assay. Since it

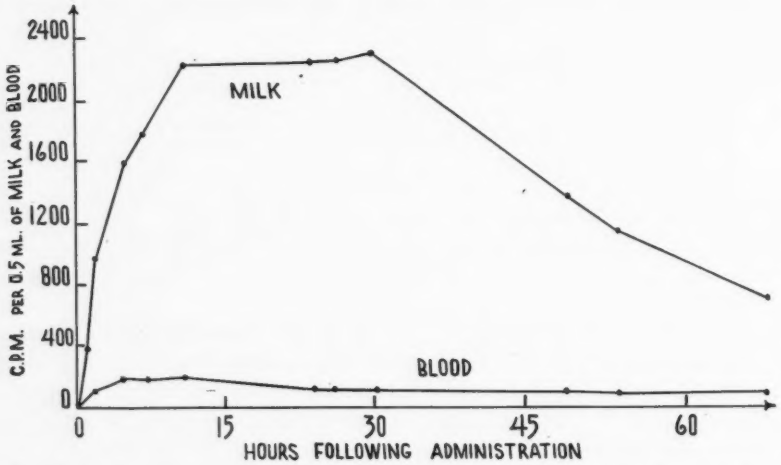

FIG. 3. Radioactivity of skimmilk and blood following the oral administration of 150 ml. of radioactive iodocasein to lactating sheep. The values represent an average of 6 ewes.

was found that there was no radioactivity present in the fat and mass absorption of the fat portion greatly interfered with the counting of samples, only the skimmed milk portion was used.

The concentration of radioactivity found in 0.5 ml. of blood and skimmilk over this period is shown in Figure 3. The values shown are average values taken from the six ewes studied. It appears that there is some concentration of iodocasein, or some fraction of same, in the mammary gland following oral ingestion of iodocasein.

Next a study of the distribution of iodine-131 in the milk was made utilizing the method previously described for making a similar study of plasma. Although many writers disclaim the presence of iodine in the milk of mammals, those who have identified it there state that it is mainly in the milk serum, which is the portion left when the fat has been removed by centrifugation and the protein removed by the addition of acetic acid

(Scharrer, 1929). This was done and the milk serum obtained was used for the distribution study shown in Table 3.

From the table it is seen that 80 per cent of the activity is associated with the milk serum. The remainder is the variable portion associated with the protein fraction. It is of significance to note that differing from blood plasma, all of the activity present in the serum fraction of milk is extractable with butyl alcohol.

Several blood samples were taken from the lambs being suckled by the ewes which had been fed the labeled iodocasein. No activity was found

TABLE 3. THE DISTRIBUTION OF IODINE-131 IN SKIMMED SHEEP MILK

Nature of material	Total activity	Activity expressed as % of skimmed milk activity
3 ml. of skimmed milk	10,002 c.p.m.	100%
Milk serum fraction	7,484 c.p.m.	75%
Butyl alcohol extract of serum	8,000 c.p.m.	80%
Thyroxine fraction of serum	3,000 c.p.m.	30%
Diiodotyrosine-inorganic iodine fraction of serum	4,972 c.p.m.	50%

there, probably due to the enormous dilution factor involved. However, the iodine compounds appearing in the milk may be the substances adversely affecting the growth of lambs suckling iodocasein treated ewes.

SUMMARY AND CONCLUSIONS

The results of the preceding experimentation would seem to indicate: (1) When iodocasein labeled with iodine-131 was injected into the jugular vein of sheep, only about 10 per cent of the radioactivity present immediately after injection could be detected in the blood 7 hours later.

(2) Iodocasein or some metabolite of it was absorbed from the alimentary tract of sheep following oral administration. There was greater absorption following oral ingestion than when the material was administered through fistulas into the ventral sac of rumen or the small intestine; there was practically no absorption from the cecum following direct administration into that region. As determined from radioactive assay, high levels of blood activity were maintained for at least 48 hours, regardless of the site of administration in the gastro-intestinal tract.

(3) A distribution study of the thyroxine, diiodotyrosine-inorganic iodine fractions of sheep plasma, showed that following oral ingestion of labeled iodocasein, 10 per cent of the radioactivity present in the plasma was found in the thyroxine fraction and 39 per cent in the diiodotyrosine-inorganic iodine fraction. Fifty per cent of the material appearing in the plasma was not extractable with butyl alcohol, indicating that iodine-131

was present in some form other than the three fractions studied. The presence of thyroxine was positively established employing the carrier technique. Similar results were obtained with rats.

(4) When labeled iodocasein was fed to lactating ewes, a radioactive assay of the skimmilk showed a considerable degree of activity. These compounds may be the substances adversely affecting the growth of lambs suckling iodocasein-treated ewes.

(5) A distribution study of the skimmed milk of ewes, similar to that carried out on the plasma, showed that 80 per cent of the iodine compounds present were extractable with butyl alcohol, 30 per cent being found in the thyroxine fraction and 50 per cent in the diiodotyrosine-inorganic iodine fraction.

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THE INFLUENCE OF THIOCARBONAMIDES AND CERTAIN OTHER SULFUR COMPOUNDS ON ALLOXAN DIABETES IN RATS[1]

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SINCE it has been known that insulin has a high cystine content and that the integrity of the disulfide linkage is essential for its physiological action, the relationship of sulfhydryl-disulfide systems to carbohydrate metabolism and diabetes mellitus has been the subject of speculation. Interest in this problem increased when it was reported that alloxan diabetes in rats could be prevented by the injection of large amounts of glutathione or cysteine intravenously very shortly prior to the intravenous administration of ordinarily diabetogenic doses of alloxan (Lazarow, 1946a). 2, 3-dimercaptopropanol (BAL) orally or parenterally also protected rats against alloxan (Chesler and Tislow, 1947). More recently, Houssay and his associates observed that the oral administration to rats of cysteine, thiouracil, and 5-methylthiouracil for twelve to thirty days also protected them from the effects of alloxan subsequently injected (1948a), and prevented the onset of diabetes in a significant proportion of partially depancreatized animals (1948b); thiourea, uracil, uramil, nucleotides, and sulfanilamide were ineffective in preventing the alloxan action. Martinez (1948) reported that the administration of these protective substances was associated with an increased sulfhydryl concentration in liver and kidney. Conversely, the enhanced sensitivity to alloxan which occurs in rats on a sodium-deficient diet (Grunert and Phillips, 1949) and of rats pretreated with ascorbic acid (Levey and Suter, 1946), is accompanied by a decrease of blood glutathione. Griffiths (1948) could induce temporary diabetes by the injection of the ordinarily non-diabetogenic uric acid in rabbits whose blood glutathione had been depleted by a diet low in cystine and methionine and high in ascorbic acid.

In general, the protective effect of substances containing the sulfhydryl group has been ascribed to the reduction of alloxan to non-diabetogenic

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dialuric acid, or, as in the case of glutathione, to the formation of an addition compound with alloxan (Patterson *et al.*, 1949).

In addition to studies of agents which modify the production of diabetes by alloxan, several recent investigations have been concerned with the effects of sulfur compounds on established diabetes. Conn and his associates (1948) observed that the impaired carbohydrate tolerance produced in human subjects during adrenocorticotrophin administration was associated with uricosuria and a decrease in blood glutathione. It was postulated, therefore, that an alloxan-like substance resulting from faulty purine metabolism might cause the temporary diabetes by reversibly inhibiting the islet cells in the presence of sulfhydryl depletion. Support for this view was presented in the report by Conn *et al.* (1949) that the intravenous injection of glutathione in a subject receiving corticotrophin resulted in a transitory fall in the elevated blood sugar concentration.

Houssay and Martinez (1948a) claimed that the administration of thiouracil cured mild, but not severe, alloxan diabetes in rats; the hyperglycemia disappeared in five of nine rats given thiouracil for twenty-three days in doses of 200 mgm./kg./day. That this effect was not mediated by the thyroid was shown by the failure of thyroidectomy in the rat to modify significantly an established diabetes (Houssay, 1948).

The intraperitoneal injection in alloxan diabetic rats of 10 mgm. of methylene blue has recently been reported (Highet and West, 1949) to induce a reduction of the blood sugar concentration, often to the normoglycemic and even hypoglycemic range.

The present study was undertaken to determine the effects on alloxan diabetic rats of certain thiocarbonamide derivatives and a few other sulfur compounds.

METHODS

Diabetes was produced in male rats (125–175 grams) of the Long-Evans and Wistar strains by the administration of alloxan monohydrate in 2 per cent solution after a 24–48 hour fast, in doses of 5–6 mgm. per 100 gm. body weight intravenously or 20 mgm. per 100 gm. subcutaneously; the resulting diabetes was generally moderate or severe, the daily glycosuria usually ranging between 5 and 12 grams glucose per day. Three to six weeks after injection the animals were placed in individual metabolism cages and daily observations were made of body weight, food and water consumption, urine volume, and urine sugar (Benedict's quantitative method). Water and food (Purina mink chow) were permitted ad libitum. Urine was collected under toluene. When the diabetes was stabilized as judged by relative constancy over several days of body weight, food consumption and glycosuria, test drugs admixed with the food were administered for periods of three to nineteen days. After the test period observations were continued until stability was again attained; the effect of each drug could then be compared with a pre- and post-experimental period. Parallel experiments were performed in the same way as these except that each rat received a daily constant suboptimal dose of protamine-zinc-insulin (0.8 μ) sufficient to decrease the glycosuria only slightly. The substances tested are listed in Table 1. A typical experiment is shown in Fig. 1.

RESULTS

Since alloxan diabetes in contrast to some other forms of experimental diabetes is apparently a pure insulin deficiency, and since replacement therapy with insulin results in weight gain coincident with alleviation of polyuria, polyphagia and glycosuria (Fig. 2A), substances which are of benefit

TABLE 1. SUBSTANCES TESTED ON ALLOXAN DIABETIC RATS. THE FIGURES IN PARENTHESES REPRESENT DAYS OF ADMINISTRATION

Compound	Dose (mgm./100 gm. body wt./day)
Thiourea	21 (10)
1,1,3-Trimethylthiourea	25 (4); 48 (9)
2-Thiouracil	18 (4); 18 (6); 21 (8); 22 (7); 38 (6)
6-n-Propylthiouracil	19 (3); 11 (5); 3.5 (3)
Dihydrothiouracil	5.4 (3)
6-Carboxythiouracil	44 (5)
6-Trifluoromethylthiouracil	12 (3)
6-Cyclopropylthiouracil	12 (4); 3 (4); 1.5 (2)
6-tert. Butylthiouracil	15.5 (4)
6-Isobutylthiouracil	17 (3)
Sodium diethyldithiocarbamate	10 (6)
2-Mercaptothiazole	8 (4); 14 (3)
4,5-Dimethyl-2-mercaptothiazole	42 (6)
2-Mercaptothiazoline	7.2 (4); 16 (5)
4-Ethyl-2-mercaptothiazoline	12 (6)
2-Mercaptoimidazole	11 (3); 18 (4); 21 (3)
2-Thiobenzimidazole	9 (4)
5,5-Diethyl-2-thiobarbituric acid	16–26 (5)
5,5-Diethyl-2,4,6-trithiobarbituric acid	17 (4)
Tetrahydro-2-thiopyrimidone	5 (3); 9 (7)
4-Phenylthiosemicarbazide	14 (3); 14 (3)
3-Phenyl-2-thiothiazolidone	19 (5)
Tetramethylthiuramdisulfide	4.9 (7); 7.5 (6); 10 (5); 12 (2)
Tetraethylthiuramdisulfide (Antabuse)	8 (8); 10 (13); 10 (1)
Tetramethylthiurammonosulfide	4.2 (3); 7 (8)
5,5-Dimethyl-2-thiooxazolidone	11 (5); 12 (6); 18 (3)
5-Vinyl-2-thiooxazolidone	12 (4); 20 (3); 18 (2); 22 (4); 11 (6); 30 (3); 25 (2)
Methylene blue	6 (4); 22.5 (4); 57 (4)
Pyridylthioacetic acid	15 (3)
Trithiomethylene	18 (3)
Cysteine hydrochloride	45 (5); 140 (7); 15 (4)
Cystine	100 (4)
Compounds tested in animals receiving suboptimal daily injection of insulin	
6-n-Propylthiouracil	2.8 (14); 3 (19)
6-Cyclopropylthiouracil	1.5 (3); 2.9 (4); 9 (5)
Cysteine hydrochloride	10–20 (9); 11 (11); 40 (4); 85 (6); 60 (5)
Thiourea	15 (11)
2-Mercaptoimidazole	12.5 (8)
5-Vinyl-2-thiooxazolidone	13.2 (6); 12.5 (5); 21 (2); 24 (3); 25 (5)
Methylene blue	30 (7)

should produce similar changes. None of the substances tested produced an insulin-like effect. In a few animals in which diabetes was becoming progressively more severe, administration of thiocarbonamide compounds failed to halt the progression. In most cases administration of the test substances was associated with no change in glycosuria or in body weight. When large doses of certain substances were used, however (e.g. cyclo-

propylthiouracil, trimethylthiourea, diethylthiobarbituric acid, methylene blue), a decreased glycosuria, polyuria and food consumption occurred, but in association with loss of body weight (Fig. 2B, C). These results illustrated the fallacy of evaluating the severity of diabetes or improvement in diabetes from a single finding such as fasting blood sugar or glycosuria.

That the magnitude of food consumption in the diabetic animal signifi-

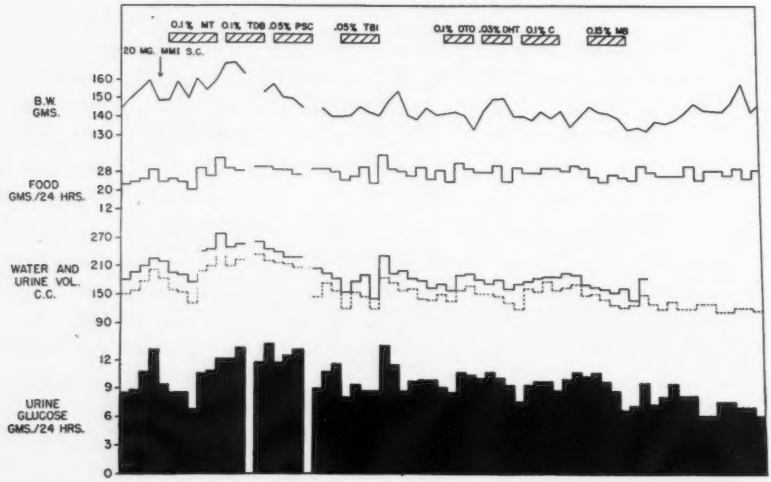

FIG. 1. The effects of feeding a number of substances to a single animal. Some of the control periods are foreshortened. The abscissa time unit is days (one day is the width of narrowest single black column). In this and succeeding charts the dotted line represents urine volume and the unbroken line just above it the water consumption. Abbreviations: MMI = methylmercaptoimidazole; MT = mercaptothiazole; TDB = trithiodiethylbarbituric acid; PSC = phenylthiosemicarbazide; TBI = thiobenzimidazole; DTO = dimethylthiooxazolidone; DHT = dihydrothiouracil; C = cysteine hydrochloride; MB = methylene blue.

cantly affects the glycosuria is not, of course, a new principle. Allen *et al.* (1919) noted that only the most severe diabetics could not be rendered aglycosuric by caloric restriction, and starvation was a standard therapeutic regimen for diabetic patients in the pre-insulin era. The results of experiments designed to study the effect of food restriction in diabetic rats are in complete accord with this concept. The amount of food daily presented to the animal was reduced, either by large, successive daily decrements of 4–5 grams or more gradually stepwise over a period of seven to ten days. It was found that on progressive limitation of the food intake on the stock diet, both the glycosuria and body weight decreased propor-

tionally to the extent of food restriction (Fig. 2D, 3). It would appear that the diminished glycosuria attendant upon the administration of some of the test substances in the diabetic animal is a reflection of reduced food consumption rather than the result of a diminution in the severity of the diabetes. It is, however, true that the weight reduction coincident with

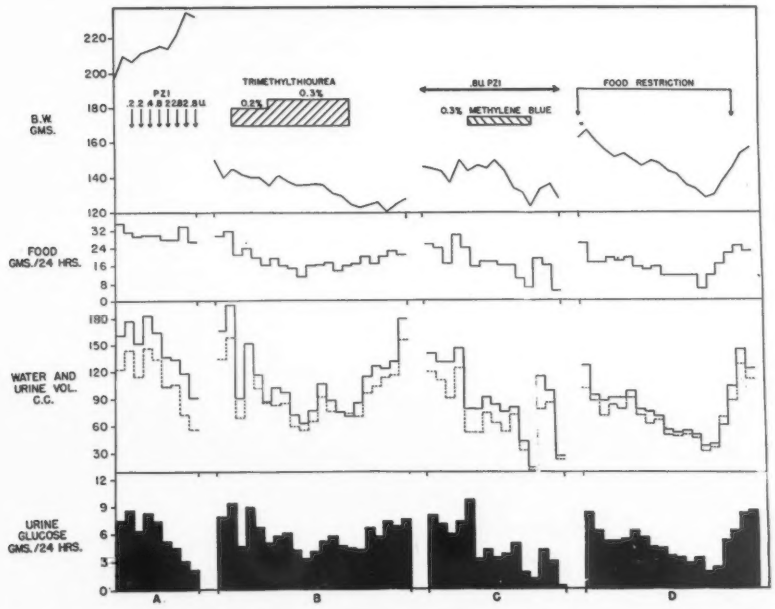

FIG. 2. Changes induced by insulin, a thiocarbonamide, methylene blue, and food restriction. Experiments performed on four different animals. A. The effect of daily injection of protamine-zinc-insulin. B. The changes associated with feeding trimethylthiourea. C. Effect of methylene blue feeding in a rat on a suboptimal insulin regimen. D. The effect of food restriction.

diminished food intake that occurred with toxic doses of certain substances was not as marked as would have been the case had food simply been restricted, and the reduction in glycosuria under these circumstances was somewhat greater than would have been expected from an equivalent degree of simple food restriction. A possible explanation lies in the fact that hungry animals whose food supply is limited tend to eat their daily ration at once while those whose intake is reduced as a consequence of ingestion of drugs in toxic or distasteful amounts eat their food more evenly distributed over twenty-four hours, the latter method being perhaps more economical of calories. Another factor that may be operative is the

depression of hypophyseal function that seems to occur in animals that are chronically ill, hypophysectomy being well known to decrease the severity of diabetes. A further possible consideration which may be involved is the apparent diminution in severity of moderate diabetes under conditions of stress observed by Ingle (1950) in depancreatized rats, where variability of caloric intake was excluded by force-feeding.

The results of administration of the test substances to rats receiving small doses of insulin differed little from those noted with animals not

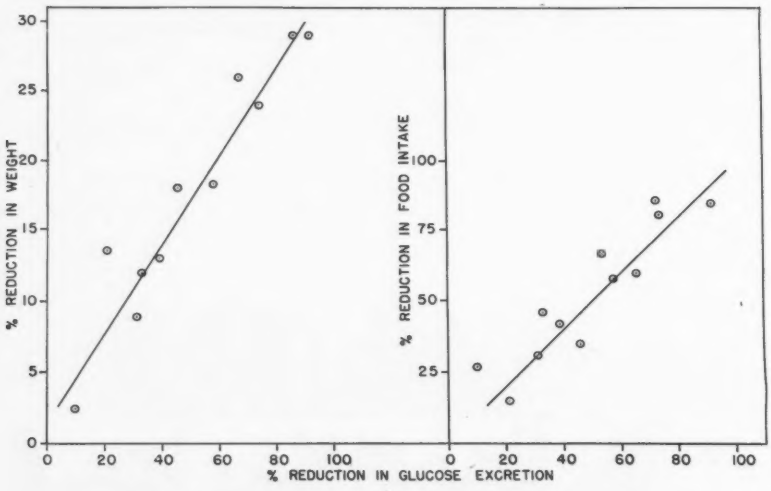

FIG. 3. Relationships between food consumption, glycosuria, and body weight on progressive reduction of food intake.

receiving insulin. No potentiation of insulin effect was observed.

Many of the substances listed in Table 1 inhibit thyroid function; in short-term experiments of a few days duration this is of little consequence the supply of pre-formed and stored thyroid hormone being sufficient to offset effects of suppression of hormone synthesis. When the experimental period is longer the antithyroid effect could become important. Thyroidectomy of rats with established diabetes induced by subtotal pancreatectomy is reported to have little ameliorating influence on the diabetes; indeed, treatment of diabetic depancreatized rats with thyroid hormone seems to have a curative action on the diabetes (Houssay, 1948). In the present experiment, it was noted in several cases that when propylthiouracil or thiourea was administered for two to three weeks, the animals' food consumption and glycosuria during the latter part of the experiment diminished moderately (up to 50 per cent) from the previous control

period, while body weight was fairly evenly maintained. However, this seemingly beneficial effect was probably more apparent then real, since when treatment was discontinued, the animals gained weight more rapidly, although glycosuria and polyphagia increased again (Fig. 4). An attempt was made, in two animals, to compensate for the antithyroid action of the test substance by feeding propylthiouracil mixed with 0.01 per cent desic-

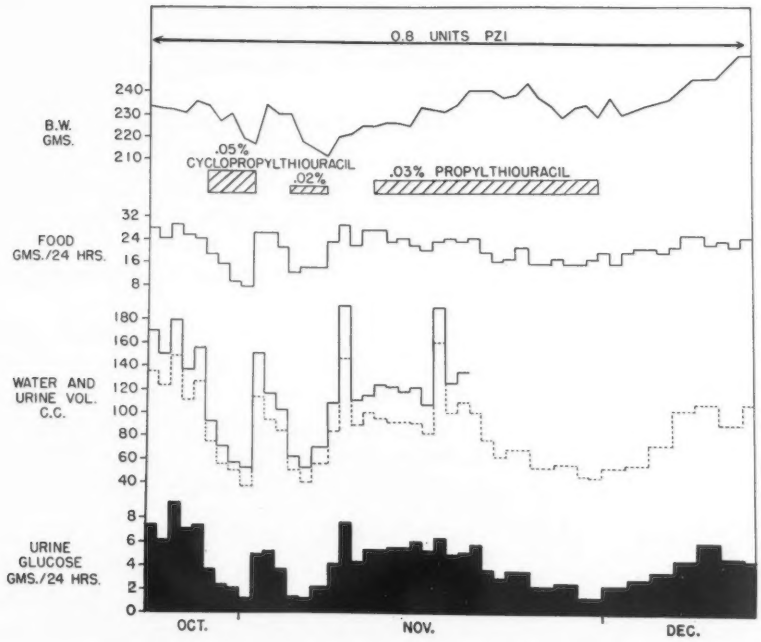

FIG. 4. Administration of propylthiouracil for 19 days to a diabetic rat on suboptimal insulin regimen.

cated thyroid powder, so that the rats received approximately their daily thyroxine requirement in the food. Neither animal showed any change in glycosuria or body weight from that in the control periods.

Several of the compounds studied were of special interest. The use of tetraethylthiuramdisulfide (Antabuse) in the treatment of alcoholism has been reported to have been associated with convulsions and coma in several diabetic patients (Gelbman and Epstein, 1949), and to have been without harmful effect in other diabetics (Lauritzen and Raby, 1949). Of three diabetic rats receiving this substance, one died, one showed no change of any kind, and the third showed a diminution in glycosuria with

weight loss and diminished food intake for several days, but on further administration reverted to previous levels of glycosuria and body weight. Feeding the closely related substance tetramethylthiuramdisulfide resulted in the death of two out of four animals and a marked weight loss in a third.

The oral administration of methylene blue in doses below 6 mgm./100 gm. body weight/day resulted in no effect, while larger doses (20–50 mgm./100 gm./day) caused weight loss, anorexia and consequent reduction in glycosuria. Intraperitoneal and subcutaneous injections of methylene blue in doses of 5–10 mgm./100 gm. body weight caused either no change in the animals or temporary (one to two days) reduction in glycosuria, usually with weight loss.

Because of the reported beneficial effects of vegetables and vegetable extracts in diabetes (Lewis, 1949), many tests were performed with *dl*-5-vinyl-2-thiooxazolidone, an antithyroid substance the *l*-isomer of which occurs naturally in the yellow turnip and other Brassica plants (Astwood *et al.*, 1949). It was found to be no more effective than the other thiocarbonamides studied in improving the carbohydrate tolerance of the alloxan diabetic rat.

Very few mildly diabetic animals were employed because these animals tend to improve spontaneously and hence lack the stability desirable in metabolic experiments. It was found possible to render aglycosuric one animal previously excreting 1 to 2 grams of glucose daily by feeding excessive doses of vinylthiooxazolidone; cessation of treatment resulted in a return of glycosuria but with an increased rate of weight gain.

SUMMARY

A number of thiocarbonamide and other sulfur compounds were fed to rats with alloxan diabetes of moderate and severe degree. No intrinsic beneficial effect on the diabetes was found, and no change in insulin sensitivity occurred.

The diminution in glycosuria which occurred during drug administration in some animals was associated with weight loss and a decrease in food consumption. Simple food restriction produced similar changes. The reduction in glycosuria in the latter instance was directly proportional to the reduction in food consumption.

Acknowledgment

The authors are indebted to Dr. E. B. Astwood for helpful suggestions.

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SUCCINIC DEHYDROGENASE LEVELS IN STRIATED MUSCLE IN RELATION TO MALE HORMONE[1]

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THERE are two well recognized instances where striated muscle has been observed to undergo marked atrophic changes following castration in the male. Papanicolaou and Falk (1938) observed this to occur in temporal muscles of the male guinea pig, and Wainman and Shipounoff (1941) indicated that similar changes occurred in the perineal musculature of the male rat. As interest in the problems of the mechanism of hormone action through modifications of intra-cellular enzyme concentration and activity has increased, it seemed that studies of pertinent enzyme systems in this type of skeletal muscle should be considered. In this report, succinic dehydrogenase was measured in the striated muscle of the perineal complex of male rats following castration, and after treatment of young males with testosterone proprionate.

Succinic dehydrogenase was chosen because other investigators had studied the levels of this enzyme in certain striated muscle following atrophy induced by other methods, namely, by vitamin E deficiency (Houchin, 1942), (Basinski and Hummel, 1947), and by denervation (Knowlton and Hines, 1934). Furthermore, since succinic dehydrogenase concentration was found to decrease in certain glandular structures (seminal vesicle and prostate) after castration in rats (Davis, Meyer, and McShan, 1949), then comparisons could be made with the response obtained from non-glandular tissue.

METHODS

Succinic dehydrogenase was determined by the method of Schneider and Potter as described in Manometric Techniques (1949). Commercial cytochrome C was employed, using 0.3 ml. of 10^{-4} M. per flask as this was found to give the maximum oxygen uptake. All determinations were made in duplicate with air as the gas phase, using 0.5 ml. of a 10% muscle homogenate in each flask. The activity of the enzyme is reported as QO_2 per mg. of dry tissue per hour. One ml. of the homogenate was dried to constant weight in a 100° C. oven to obtain the dry residue upon which the calculations were based.

Adult, young adult and pre-pubertal male rats of the Long-Evans strain were employed. In the rats which were castrated, a period of 30 days was allowed to elapse before assaying the muscles. In one experiment, normal rats 40–45 days old were injected with 1 mg. of testosterone proprionate per day for 10 days.[2]

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[2] Acknowledgement is made to the Schering Corporation for the testosterone proprionate used in these experiments.

The perineal complex was removed quickly after killing the rats by a blow on the head and severing the neck to permit bleeding. An incision was made medially through the scrotum and extended anteriorly beyond the symphysis pubis. The penis was dissected free from the skin and fascia as far as the pubis. The fat and fascia between the scrotal sacs were dissected to expose the perineal muscles and rectum. The symphysis pubis was cut in the mid-line and the halves of the pelvis spread apart. The ischio-cavernosus muscles were cut at their attachment to the pelvis, and the urethra was cut a short distance anteriorly to these muscles. By pulling upward on the penis, the perineal muscle complex was easily dissected free as a unit, by severing the rectum on both sides of the levator ani muscle. All further removal of the connective tissue and dissecting of the muscles were carried out on a piece of moistened filter paper resting on an ice tray.

Removing the muscles for assay presented some difficulty in the young castrated animals since it was impossible to exclude all the connective tissue. The muscles comprising the perineal complex used in this study were, the *levator ani*, the *ischio-cavernosus*, and the *bulbo-cavernosus*. In the center of the bulbo-cavernosus runs a core of tough connective tissue. In normal rats and adult castrates, this was relatively easy to dissect out, but in the young castrates it was impossible, and a high percentage of connective tissue was included with these muscles. Superficial fascia, fat and the Cowper's glands were removed without difficulty. After weighing the combined muscle tissue on a torsion balance, a 10% aqueous homogenate was immediately prepared in an ice bath and retained there until ready for use. It was necessary to pool the muscles of 3 of the young castrated rats to obtain sufficient tissue for assay.

It was recognized that the weights of the perineal muscles was only a close approximation of the total from each rat because of the difficulties in dissection. However, since all operations were performed by one individual, the actual values give an index of the weight changes which occurred in the perineal complex following the various experimental procedures.

RESULTS

In the first experiment, succinic dehydrogenase was determined in the perineal muscles of 3 groups of young males 30 days following castration. A control animal was run simultaneously. As seen in Table 1, the QO_2 for succinic dehydrogenase in the muscles of the castrates was in every instance lower than that in the normal control, but in only 3 cases in 10 was this very marked. These rats were not fully matured, since in every instance, the seminal vesicles of the control animals at autopsy were not fully distended with fluid. It was also noted that the effect of castration was so marked that the total muscle dissected from 3 castrated rats did not equal in weight that obtained from the respective control.

The technical difficulties of recovering sufficient muscle tissue from these young males, coupled with the fact that a large amount of connective tissue was unavoidably included in the homogenate, led to the opinion that the low results in castrates were not a true indication of the enzyme concentration. For this reason fully mature males were used in the next experiment, and after a similar period of castration, assays were made as before. Table 1 shows that no difference was found in the succinic dehydrogenase levels between the castrated and control rats. It was relatively easy to

TABLE 1. SUCCINIC DEHYDROGENASE IN PERINEAL MUSCLES OF MALE RATS

Experiment	Castrated males			Control males			% difference
	Body wt., gm.	Muscle wt., mg.	SDH-ase QO_2	Body wt., gm.	Muscle wt., mg.	SDH-ase QO_2	
Young males[1] 63–67 days old	196	158	5.0	227	363	9.6	−48
	189	172	5.9	249	340	7.5	−21
	214	183	7.5	248	456	10.1	−26
	219	178	7.6	252	379	9.1	−16
Young males[1] 69–73 days old	208	226	10.6	230	327	12.9	−18
	212	224	12.7	210	402	14.4	−12
	205	180	11.5	203	380	14.1	−18
Young males[1] 98–103 days old	263	220	4.6	254	440	10.0	−54
	269	307	4.1	302	657	8.8	−53
	267	290	5.8	320	540	7.7	−25
							Av. −29
Adult males 5–6 months old	380	303	9.7	366	631	8.5	
	376	363	9.4	428	904	6.3	
	378	530	7.2	354	1032	8.3	
	378	401	7.7	325	970	9.9	
	388	344	7.0	400	1001	9.2	Av. −2
	330	374	6.9				
	300	305	8.8				
	350	355	8.9				
			Av. 8.2			Av. 8.4	
	Normal injected			Normal controls			
Young males 50–55 days old given testerone for 10 days	156	341	17.8	206	355	16.7	+ 7
	157	394	13.9				−17
	162	421	16.8	188	332	18.4	− 9
	162	407	19.4				+ 5
	158	383	19.2	225	350	18.3	+ 5
	158	421	19.4				+ 6
	176	389	18.3	174	236	15.8	+16
							Av. +2

[1] The body weights of the Castrates is an average of 3 rats; the muscle weights represent the total from 3 rats.

secure enough tissue from one of these 30-day adult castrates without including the central core of connective tissue. It was noted that even though the muscle mass was reduced by 1/2 to 1/3 of the normal amount, the enzyme concentration remained normal.

If succinic dehydrogenase concentration in this muscle complex was controlled by the male hormone, then it seemed logical to expect a change in the enzyme concentration if immature males were treated with androgens. A group of young males were treated for 10 days with testosterone proprionate, and the muscles assayed. At autopsy, the seminal vesicles of the injected rats were enormously swollen with fluid while the control glands were very small. Succinic dehydrogenase levels of the muscles of the experimental and control animals were approximately the same (Table 1). The perineal muscles did not enlarge to any appreciable extent in this period of treatment but they were of such size that a good sample of muscle

could be obtained without the interfering connective tissue.

In order to compare the effects of castration upon other skeletal muscle of the body, samples of the dorsal thigh muscles trimmed of connective tissue were taken from normal and 30-day castrates (2 to 4 months old) and assayed for succinic dehydrogenase. The average QO_2 for the thigh muscles of 5 normal rats was 26.5 (range 22.3–29.9) and that for 5 castrates was 25.9 (range 21.2–30.6). Thus no change in enzyme concentration was noted. The succinic dehydrogenase levels of the skeletal muscles of the thigh were greater than that of the perineal muscles.

Another observation, made incidental to this study, should be mentioned. It was noted that a definite trend to a higher level of succinic dehydrogenase occurred in the perineal muscles of young males. In 6 old adult males, over 8 months of age, the enzyme concentration measured as QO_2 was 7.0 (range 6.6–7.9) which was less than half that found in young males 55 days of age.

DISCUSSION

Although the accessory glands of reproduction and perineal striated musculature of the male rat were influenced morphologically by the levels of androgens, no alterations in the succinic dehydrogenase concentration occurred in the perineal muscles as a result of castration or hormone treatment. On the other hand, the seminal vesicles and prostate are reported to show decreased amounts of enzyme as a result of castration and the enzyme levels are returned to normal by administration of androgens (Davis, Meyer and McShan, 1949). The apparent contradictory results of the effect of castration on the muscle enzyme levels in some of the young males seemed to rest with technical difficulties in obtaining a relatively pure muscle sample of sufficient size to make the determinations.

Further evidence that there may be little if any difference in the oxidative metabolism of the perineal muscles between normal and castrated males can be noted from the results of Eisenburg, Gordon and Elliot (1949). They observed no difference in oxygen consumption (total respiration) in strips of these muscles from young male rats. However, testosterone *in vitro* was observed to inhibit oxygen uptake in the *levator ani* muscles from both the normal and castrated rats.

Atrophy of a leg muscle (gastrocnemius) of the rat, induced by severing the nerve, was accompanied by a decrease in concentration of succinic dehydrogenase by as much as 55% one week after denervation (Knowlton and Hines, 1934). These investigators could observe no difference, however, in the rate of O_2 consumption (respiration) of this muscle under similar experimental conditions. It would appear that atrophy of the striated muscles of the perineal region induced by castration was physiologically different than that caused by denervation of the leg muscle.

Atrophied skeletal muscles of the hamster (thigh muscle) induced by feeding diets deficient in α-tocopherol were studied for changes in succinic dehydrogenase levels by Houchin (1942) who reported an increase in the enzyme concentration following atrophy. Basinski and Hummel (1947), however, from the same laboratory, were unable to confirm this. The latter investigators also showed that succinic dehydrogenase concentration of thigh muscle decreased with age in this species.

The enzyme concentration in the perineal muscles of the rat was also higher in the younger animals than in the older ones. The difference was more striking here than that reported for the leg muscles of the hamster. Recently, Gaebler, Mathies and Palm (1949) studied the effect of growth hormone on succinic dehydrogenase concentration in the *tibialis anticus* muscle of rats and found that the enzyme concentration remained constant and in proportion to the increase in muscle mass. This might hold true for short periods of time with such treatment but the evidence cited above would indicate that even though the mass of muscle may be increased, with advancing age, a decrease in the concentration of this enzyme will eventually occur. Skeletal muscle taken from the thigh of the rat was higher in enzyme concentration than that found in the perineal muscles which may be associated with the greater activity of the former.

SUMMARY

Castration of adult male rats caused no change in the succinic dehydrogenase levels of the striated perineal musculature which had undergone atrophy as the result of the loss of androgens. A small, consistent decrease in succinic dehydrogenase concentration was observed in young castrated males, which is interpreted as being inconsequential because of the unavoidable inclusion of excessive connective tissue. Testosterone proprionate treatment of young male rats for 10 days failed to alter the enzyme concentration. With increasing age, there was a decrease in the concentration of the enzyme in the perineal muscles. Thigh muscles of the rat have a higher concentration of succinic dehydrogenase than that of the perineal muscles, but they likewise showed no change in enzyme concentration as a result of castration.

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THE INACTIVATION OF ESTRONE BY RATS IN RELATION TO DIETARY EFFECTS ON THE LIVER[1]

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THE recognition of a relationship between the nutritional status of the animal and the ability of the liver to inactivate estrogen was initiated by Biskind and Biskind (1942). These authors reported that the ability to inactivate estrogen coming to the liver from an intrasplenic pellet was lost when the rats were placed on a diet deficient in the vitamin B complex. Other investigators (Shipley and Gyorgy, 1944; Segaloff and Segaloff, 1944; Singher *et al.*, 1944) obtained confirmatory data with various vitamin deficient diets, especially thiamine and riboflavin deficiencies. These results were shown by Drill and Pfeiffer (1946) and later by Jailer (1948) to be due to the decreased food intake that accompanies an acute deficiency of these particular vitamins. It has further been shown that the inanition effect is due to a decreased protein intake rather than a caloric restriction (Jailer, 1950). The importance of an adequate protein intake in the diet was first demonstrated by Unna *et al.*, 1944, who found that liver slices from rats maintained on an 8% casein diet were unable to inactivate estrogen.

The effects of acute inanition and a low protein diet on various liver stituents have been found to be somewhat similar; the effect of a low protein diet is potentially more severe because it can be carried on for a longer period of time. Both cause a rapid loss of protein (Addis *et al.*, 1936), ribonucleic acid and phospholipid (Campbell and Kosterlitz, 1947; Kosterlitz, 1947); riboflavin is also lost on a low protein diet (Sarett and Perlzweig, 1943; Unna *et al.*, 1944; Griffin and Baumann, 1946). The effect of inanition has been studied on xanthine oxidase and a few other liver enzymes (Miller, 1948; Rosenthal, 1937; Lightbody and Kleiman, 1940; Wachstein, 1945). So far only one liver enzyme, xanthine oxidase, has been shown to be severely depleted by feeding a low protein diet (Westerfeld and Richert, 1949; 1950). Glutathione appears to be another liver constituent that suffers a severe loss from either starvation or a low protein diet (Leaf and Neuberger, 1947).

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[2] Bristol Laboratories Fellow.

TABLE 1. THE EFFECT OF DIET ON LIVER XANTHINE OXIDASE AND THE ABILITY OF THE LIVER TO INACTIVATE MESENTERICALLY IMPLANTED ESTRONE

Type of experiment	No. of rats	Food intake gm./day	Body weights in gms.		Vaginal smears		Uterine weight, mgm.	Activity in cmm. O^2/gm. dry wt./hr.		
			Initial	Final	Days negative	Days positive		Liver xanthine oxidase	Liver d-Amino acid oxidase	Kidney d-Amino acid oxidase
Normal Males on chow	6	—	—	314 ± 4.4	—	—	—	1275 ± 131	2580 ± 372	16,620 ± 1735
Normal Females on chow	13	—	—	235 ± 9.9	—	—	351 ± 25.2	1595 ± 102	2710 ± 259	16,930 ± 800
Castrate plus Pellet on chow*	13	—	—	218 ± 7.4	0	39 ± 2.0	266 ± 23.6	1270 ± 122	2385 ± 339	17,600 ± 825
Castrate plus Pellet on low-protein diet	10	14.2 ± 0.4	198 ± 4.4	215 ± 6.6	17 ± 2.1	6 ± 0.3	222 ± 17.6	178 ± 93	2220 ± 172	16,165 ± 1317
	7	14.1 ± 0.6	200 ± 6.4	220 ± 8.9	30 ± 3.7	0	198 ± 4.5	100 ± 64	2355 ± 962	17,280 ± 1000
Castrate plus Pellet on Restricted Chow Diet	8	8.5 ± 0.3	223 ± 6.3	178 ± 5.4	15 ± 1.5	4 ± 1.8	276 ± 8.3	1245 ± 112	2050 ± 310	16,275 ± 492
	4	9.0 ± 0.3	210 ± 9.1	173 ± 9.2	17 ± 1.2	0	158 ± 34.4	1060 ± 208	2055 ± 316	15,675 ± 142
Normal Females on low-protein	8	16.3 ± 1.8	192 ± 5.2	233 ± 4.8	Normal cycles for >56–102 days		440 ± 46.0	60 ± 52	1970 ± 174	13,080 ± 684
Normal Females on restricted chow	8	7.7 ± 0.3	190 ± 2.7	153 ± 6.7	Anestrus in 11 ± 1.0 days; sacrificed after 35 days		113 ± 9.1	1390 ± 78	1915 ± 93	15,820 ± 667

All values are mean ± standard error.
* In continuous estrus, presumably from faulty implantation of the pellet.

This study was designed to see if the effect of a low protein diet on estrogen inactivation in the liver was due to a depletion of liver xanthine oxidase. Such a relationship was considered within the realm of possibility because xanthine oxidase is known to produce hydrogen peroxide during the aerobic oxidation of its substrates, and hydrogen peroxide is capable of inactivating the estrogens. However, the evidence showed that the two systems could be affected independently, and the enzyme(s) responsible for estrogen inactivation could be considered to be a separate liver constituent that, like xanthine oxidase, was also seriously depleted by a low-protein diet. Further evidence of the effect of inanition and a low-protein diet on gonadal function in the normal rat was also obtained.

EXPERIMENTAL

Adult Albino female rats weighing 150–225 gms. were ovariectomized and implanted with a pellet of estrone[3] (7–13 mg.) in the mesentery according to the method of Burrill and Greene (1942). Estrone was used in these studies instead of estradiol in order to avoid the possibility that "inactivation" of the latter merely represented its conversion to estrone with a resulting 8–10 fold decrease in activity. Following a control period of anestrous smears for 6–10 days, during which the rats were fed Purina dog chow *ad libitum*, the experimental diets were started. Vaginal smears were followed daily until the experiment was terminated. Daily food consumptions and body weight changes have been recorded in the tables. At autopsy the uterus was weighed after expressing the fluid, and the liver and kidney were analyzed for xanthine oxidase and d-amino acid oxidase.

Liver xanthine oxidase was determined by the method of Axelrod and Elvehjem (1941) as previously described (Richert, Edwards and Westerfeld, 1949). Liver and kidney d-amino acid oxidases were determined by the method of Axelrod, Sober and Elvehjem (1940) to ascertain the effect of these dietary procedures upon another flavoprotein enzyme.

In addition to chow, which is 21% protein, some of the rats were fed *ad libitum* the purified 8% casein and 21% casein diets previously described (Westerfeld and Richert, 1950) but substituting sucrose for glucose. In some experiments the intake of the chow and 21% casein diets was restricted to approximately 50% of that eaten *ad libitum* during a control period of one week immediately preceding the inanition study.

RESULTS

Table 1 records the liver xanthine oxidase and d-amino acid oxidase activities in rats maintained on 1) chow *ad libitum*, 2) low protein (8%

[3] The estrone pellets were furnished through the courtesy of Dr. Fred Houghton, Ciba.

casein) *ad libitum*, and 3) 50% restricted intake of chow. The effect of each dietary regime on the vaginal smear, hence on the estrone inactivating ability of the liver, is also given.

Normal males and females maintained on chow both had about the same liver xanthine oxidase. The values were not changed significantly in castrate females kept in continuous estrus by the pellet; failure to inactivate the pellet estrogen by normal rats on chow was observed in about 1/3 of the operated animals, and was presumably due to a misplaced pellet or to the establishment of a collateral circulation to the pellet or to the overwhelming absorption of estrogen from the pellet. Such animals, of course, could not be used for the dietary studies. An adequate estrogen inactivating system was maintained by the chow diet *ad libitum* (which furnished 3.56 gm. protein per rat per day) in all rats whose diets were subsequently varied.

Pellet implanted rats placed on the low-protein diet *ad libitum* received 1.13 gm. protein per day, and developed positive smears after an average 17 days; within this period of time the liver lost its ability to inactivate the estrone coming to it from the pellet. An 8% gain in body weight during the low-protein regime showed that inanition was not a factor in this response. After 6 days of continuous positive smears the rats were sacrificed for enzyme analyses. As expected, the liver was depleted of its xanthine oxidase as a result of the low-protein intake. The independent nature of these two low-protein effects was suggested by other rats which were sacrificed as controls while they still had negative smears. In these rats the livers also were depleted of xanthine oxidase, but the negative vaginal smears indicated an adequate estrogen inactivating ability. These results were inconclusive, however, because the negative smears could possibly have been due to a failure in pellet absorption.

Further proof that the estrogen inactivating system could be affected independently of liver xanthine oxidase was obtained in a group of rats whose intake of chow was restricted to approximately 50% of that eaten *ad libitum*. These rats consumed 1.78 gm. of protein per day and lost their ability to inactivate estrone in an average 15 days and in the absence of any significant decrease in liver xanthine oxidase; body weight losses averaged 26%. A similarly treated group of rats that were still showing negative smears at 17 days also had liver xanthine oxidase levels that were not seriously depleted. The results suggest that liver xanthine oxidase is equally or more sensitive to a low-protein diet than is the estrogen inactivating system, while the latter is more sensitive to a restricted food intake.

Normal rats maintained on the low-protein diet continued to have normal estrus cycles for at least 56–102 days (the duration of the experiment). The estrogen inactivating system was presumably depleted in at least

some if not all of these rats, and it can be noted that the uteri were somewhat enlarged. However, the periodic production of estrogen was not disrupted, and there was no continuous effect from the estrogen so produced. Zondek and Finkelstein (1947) showed that injected estrogen was destroyed in the rat even when the liver inactivation system had been depleted by dietary means.

Normal rats fed 50% of their usual intake of chow developed an anestrus smear in an average 11 days which persisted for at least 23 days. Food restriction therefore stopped the cyclical production of estrogen as well as its destruction by the liver.

Liver and kidney d-amino acid oxidases were not grossly altered by either a low-protein diet or a restricted food intake.

The effect of a purified 21% casein diet was studied in a group of 7 pellet implanted rats whose estrogen inactivating system was first depleted by feeding 50% of their *ad libitum* intake of chow for about 5 weeks. The 21% casein diet fed *ad libitum* furnished 3.28 gm. protein per day, and restored the estrogen inactivating system in an average of 14 days (Expt. I, Table 2). The same group of rats was then limited to a 50% intake of the 21% casein diet. Positive smears were not obtained in 70–93 days. When the food consumption of this group was reduced to 25% of the *ad libitum* intake, positive smears appeared in an average of 19 days; negative smears were restored in 14 days when the food intake was again returned to the 50% level. The functioning of the estrone pellet was then verified by placing the rats on the restricted chow diet, which again resulted in positive smears in all cases.The adequacy of a 50% intake of the 21% casein diet was verified in another group of 6 pellet implanted rats which was started directly on this regime after a preliminary depletion period with restricted chow (Expt. II, Table 2). The 50% intake of the 21% casein diet restored the estrogen inactivating system in an average of 18 days. While these results discredit inanition per se as a major factor in the 50% chow response, they are not conclusive because of the smaller body weight loss with the casein diet.

The only difference between the adequate 21% casein diet and the inadequate 8% casein diet was the isocaloric substitution of casein for sucrose. The *ad libitum* feeding results show that the dietary factor involved in maintaining the estrogen inactivating system in the liver is either protein or some unidentified factor carried on the vitamin-test casein. The adequacy of a 50% intake of the 21% casein diet as compared with a similarly restricted in take of chow suggests that casein is a better protein for this purpose than the mixture of proteins found in chow, or it is a better carrier of the unidentified factor. If the effect were due to protein, as seems likely, the critical level of casein necessary to maintain the estrogen inactivating

TABLE 2. THE EFFECT OF A PURIFIED 21% CASEIN DIET ON THE ABILITY OF THE LIVER TO INACTIVATE MESENTERICALLY IMPLANTED ESTRONE

Expt.	Diet	Food intake, gm./rat/day	Vaginal smears		Body weights, gms.
			Positive or negative	Days on diet	
I	Chow ad libitum	15.6 ±0.3	−	—	231 ±11.4
	Restricted chow	7.8 ±0.1	+	36 ±10.8	172 ± 9.3
	21% Casein	15.6 ±0.3	−	14 ± 4.9	241 ± 9.8
	Restricted 21% casein	7.8 ±0.1	−	70 ±10.8	197 ± 6.9
	Restricted 21% casein*	3.9 ±0.1	+	19 ± 2.2	144 ± 5.2
	Restricted 21% casein	7.8 ±0.1	−	14 ± 2.9	184 ± 8.3
	Restricted chow	7.8 ±0.1	+	42 ±10.2	164 ± 8.9
II	Chow ad libitum	17.2 ±0.8	−	—	238 ±17.5
	Restricted chow	8.6 ±0.4	+	40 ±11.5	180 ±14.7
	Restricted 21% casein	8.6 ±0.4	−	18 ± 4.1	196 ±13.4
	Restricted chow	8.6 ±0.4	+	25 ± 4.6	182 ±10.0

All values are mean ± standard error.
* Two animals died on this regime from starvation.

TABLE 3. THE EFFECT OF A 50% RESTRICTED CHOW INTAKE FOR 35 DAYS ON PITUITARY GONADOTROPIN IN NORMAL FEMALE RATS

No. of rats	Body weight (gms.)		Chow diet	Organ weights at autopsy					Recipient Mice (1 pituitary/mouse)		
	Initial	Final	gms./day/rat	Adrenals, mg.	Ovaries, mg.	Uterus, mg.	Thyroid, mg.	Pituitary, mg.	Body weight, gm.	Uterus, mg.	Ovaries, mg.
6	120±3.6	187±14.5	Ad libitum	39.6±2.8	87.8± 5.4	289 ±39.6	10.0±1.1	6.1±0.8	10.8±0.4	20.4±5.6	6.8±0.5
6	189±9.5	139± 8.1	6.0±0.2	32.0±1.2	37.5± 1.6	112 ± 7.4	7.3±0.9	6.7±0.6	10.2±0.5	26.1±4.9	8.5±0.8
6*	185±8.4	140± 2.5	6.2±0.2	30.8±1.3	43.7± 2.7	160.7± 4.1	7.7±0.6	6.3±0.4	12.2±0.7	21.0±2.0	7.4±0.8
							Saline	Controls	11.5±0.5	8.5±0.8	6.4±0.3
10	188±4.8	206± 5.7	Ad libitum	48.8±1.8	77.2±12.5	394.0±17.6	10.5±0.6	11.6±0.5	13.3±0.6	26.5±1.0	7.3±0.6
10	188±5.2	122± 5.5	7.6±0.3	34.8±2.0	35.8± 2.7	98.0± 4.5	7.0±0.4	6.5±0.3	13.5±0.6	31.9±1.1	8.3±1.1
10*	187±4.8	124± 6.8	7.6±0.3	33.1±1.6	36.5± 2.6	156.7± 6.3	6.8±0.5	6.4±0.3	11.8±0.4	26.5±1.8	7.9±0.7
							Saline	Controls	12.7±0.5	7.8±0.6	4.6±0.3

All values are mean ± standard error.
* Injected with 0.15 gamma diethylstilbestrol on days 31, 32 and 33.

system in the rat appears to be between 8% and 10.5% of the diet, though it should be emphasized that the latter figure was obtained on a restricted caloric intake.

Inanition vs. Pituitary Gonadotropin

The cessation of estrus cyles in normal rats fed half their usual intake of chow was due to pituitary dysfunction during inanition. Maddock and Heller (1947) and Rinaldini (1949) found that the gonadotropin content of the pituitary increased during inanition, but that the release of these hormones into the circulation was restricted.

Two similar experiments were carried out in which the pituitary gonadotropin was measured in three groups of normal female rats fed: 1) chow *ad libitum* for 35 days, 2) and 3) 50% of the usual intake of chow for 35 days. The first two groups received 0.15 cc, of sesame oil subcutaneously on days 31, 32, and 33, while the third group received the oil containing 0.15 gamma diethylstilbestrol. At autopsy on the 35th day the endocrine organs were weighed; the pituitaries in each group were pooled, homogenized in saline, and the equivalent of one rat pituitary was injected over a period of 3 days into each recipient mouse according to the method of Smith (1944).

The results are shown in Table 3. While the differences in pituitary gonadotropins were relatively small by this assay, they were consistent and in confirmation of the earlier work. Inanition increased the pituitary gonadotropin at the same time that the ovaries and uterus atrophied. Others (see Rinaldini, 1949) have demonstrated that under these conditions the ovary is capable of responding to injected gonadotropins and the uterus is capable of responding to injected estrogens. Uterine stimulation by the injected diethylstilbestrol was observed in these experiments. The results also suggest that injected diethylstilbestrol released some of the excess pituitary gonadotropin, but the original differences were too small to draw such a conclusion with confidence.

DISCUSSION

Biskind (1946) attempted to relate certain clinical conditions, such as excessive uterine bleeding and cystic mastitis, to an inadequate intake of the B-vitamins. The relationship of these two conditions to abnormal liver function and to the nutritional status of the patient, particularly with reference to the protein intake, would seem to merit further study. Normal rats on a low-protein diet continued to have estrus cycles, but uterine weights indicated a greater estrogenic stimulation than occurred in rats on a normal diet. Impaired liver function in humans, whether arising from a similar dietary defect or from other causes, could theoretically be the

fundamental defect that gives rise to cystic mastitis or to the hyperplastic endometrium so often associated with functional bleeding. The primary cause of the prolonged estrogenic stimulation and the absence of progesterone production which gives rise to a hyperplastic endometrium has yet to be determined.

SUMMARY

The estrogen draining through the liver from mesenterically implanted pellets of estrone in castrate rats was inactivated when the rats were maintained on: 1) chow *ad libitum*, 2) normal or 50% restricted intake of a 21% purified casein diet. The liver estrogen inactivating system was not maintained by: 1) an 8% purified casein diet *ad libitum*, or 2) 50% of the usual intake of chow. The results are consistent with the concept that protein intake is the critical dietary factor involved in maintaining the estrogen inactivating system in the liver.

The effect of diet on the estrogen inactivating system was independent of its effect in depleting liver xanthine oxidase. Both were depleted on a low-protein diet, but only the estrogen inactivating system was seriously depleted on a 50% chow intake. Liver and kidney d-amino acid oxidases were not affected appreciably by either a low-protein diet or food restriction.

Normal estrus cycles were maintained on a low-protein diet, but were stopped in anestrus on a 50% chow restriction. Pituitary gonadotropin increased slightly during inanition.

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THE NUCLEIC ACID CONTENT AND SUCCINIC DEHYDROGENASE ACTIVITY OF STIMULATED PIGEON CROP GLAND TISSUE[1]

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NUMEROUS investigators have found that rapidly growing tissues have a high content of nucleic acids and the relationship of these acids to the synthesis of cellular proteins has been discussed in recent reviews (Caspersson, 1947; Thorell, 1947; and Davidson, 1949). In the light of these results the rapid growth of tissues which are stimulated by hormones presumably would also show an increase in nucleoprotein and nucleic acids. The growth of these tissues is often associated with an increase in the activity of enzymes which in turn can be correlated with functional changes that accompany the growth. Thus there is an increase in succinic and malic dehydrogenases during the growth of hormonally-stimulated tissues such as the corpus luteum of the rat during pregnancy (Meyer, Soukop, McShan and Biddulph, 1947; McShan, Erway and Meyer, 1948), and the growth of the prostate and seminal vesicle of the rat under the influence of androgen (Davis, Meyer and McShan, 1949).

It was of interest, therefore, to determine whether the growth and functional changes that occur when a tissue is stimulated by a hormone are correlated with an increase in nucleic acids and with changes in the activity of certain enzymes. The crop gland of the pigeon was selected for study since it grows rapidly and produces the so-called "crop-milk" under the influence of pituitary lactogenic hormone. The changes in desoxypentose nucleic acid (DNA) and pentose nucleic acid (PNA), and the activity of succinic dehydrogenase were determined during the growth and secretory processes initiated by the action of the hormone.

MATERIALS AND METHODS

The pigeons used were wild birds obtained from various sources and kept in the vivarium for three weeks or longer before use. They were fed a diet of cracked grain. Their body weights ranged from 200 to 500 grams at autopsy, but most of the birds weighed between 300 and 400 grams.

The lactogenic hormone preparations used were prepared from sheep pituitary glands by the method of Lyons (1937). The preparations were prepared for injection by dissolving in 0.9 per cent saline. Solution was effected by addition of dilute NaOH after which the solution was adjusted to pH 7 with dilute HCl.

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The solution used for the first group of birds was diluted to the proper volume with saline so that the concentration was 0.5 mg. per ml. Each bird received 0.25 ml. of the solution or 0.125 mg. of the preparation per injection. The injections were made on the morning and evening of each day for a period of 1 to 5 days. There were 33 birds in the first injected group; eight others were kept for controls and received no treatment. The treated birds were killed 12 hours after the last injection on days 1, 2, 3, 4, and 5.

A second group of 10 birds received two injections per day for four days. A different hormone preparation was used for these injections which contained only half the activity of the above preparation. Consequently the concentration of this solution had to be doubled. Two birds were killed on the morning of day 1, four on the morning of day 4, and four on the morning of day 7 after the last injection.

The animals were killed by decapitation and the crops were removed. An attempt was made to standardize the dissection so that each crop would be severed from the esophagus at approximately the same point. The tissue was freed from fat and washed under the tap. After blotting with filter paper to remove excess water, the whole crop was weighed. Tissue samples for the determinations were taken from the central portion of the lateral glandular lobes.

Nucleic Acids. The procedure used for the determination of nucleic acids was essentially that reported by Schneider (1945a). The exact procedure employed is as follows: The tissue (200–400 mg.) was ground thoroughly in a sharp-pointed glass homogenizer[2] with a minimum quantity of distilled water. To this homogenate was added 2.5 volumes of cold (4° C.) 10% trichloroacetic acid. The tubes were centrifuged and the supernatant was measured in graduated tubes. The precipitate was washed once in 5% trichloroacetic acid (2 ml./200 mg.), centrifuged and the supernatant added to the first extract. The total volume of the extract was recorded and was considered to contain all of the nucleic acids of the original tissue.

PNA was determined by the orcinol method described by Mejbaum (1939) as recommended by LePage and Umbreit (1945). DNA was determined by the diphenylamine reaction of Dische (1930) as modified by Schneider (1945b).

As a basis for calculating the amounts of DNA and PNA present in the experimental tissues, certain analytical values for purified samples of nucleic acid[3] were determined according to Schneider (1945a). Stock solutions of two samples each of DNA and PNA were prepared containing 1 mg. nucleic acid/ml. The diphenylamine reaction was carried out on the two solutions of DNA and the orcinol reaction on all four solutions. The L value (log 100 minus log transmission) obtained by use of an Evelyn photoelectric colorimeter was determined per ml. of stock solution. The phosphorus content/ml. of stock solution was determined using the method of Fiske and Subbarow (1925) and the L value/ml. for orcinol and diphenylamine was divided by the μg. P/ml. to give an L value/μg. P. The average L value/μg. P for orcinol and PNA was 0.00495; for orcinol and DNA, 0.00506; and for diphenylamine and DNA, 0.00797. These constants were then used to calculate the nucleic acid content of the unknown extracts in the following manner:

Calculation of DNA using one ml. of extract. (P content of DNA taken as 9.9%.)

$$\frac{\text{L diphenylamine}}{0.00797} = \text{A} \qquad \frac{\text{A}}{0.099} = \mu\text{g. DNA/ml. of extract}$$

[2] This type of homogenizer is made by Norman D. Erway, Oregon, Wisconsin.

[3] The authors are indebted to Dr. W. C. Schneider of the National Institute of Health, Bethesda, Maryland for the purified samples of nucleic acids.

Calculation of PNA. (P content of PNA taken as 9.5%.)

$$\text{L orcinol} - (A \times C) = B$$

$$\frac{B}{0.095} = \mu\text{g. PNA/ml. of extract}$$

$$C = \frac{\text{L orcinol (DNA)}}{\mu\text{g. P}} = 0.00506$$

Purified samples of nucleic acids usually contain some nitrogenous impurities but Schneider (1965b) found that the ratio of P to ribose was fairly constant. Hence P content was used as the basis for calculation.

Succinic Dehydrogenase. Crop gland tissue was rinsed in ice-cold glass-distilled water, blotted dry on analytical grade filter paper, weighed (approx. 100 mg.), homogenized and diluted with glass-distilled water to make a 5% homogenate. The composition of the reaction mixture and exact procedure used for the determination of succinic dehydrogenase have been described elsewhere (Schneider and Potter, 1943; McShan, Erway and Meyer, 1946). A second piece of tissue weighing 100 to 200 mg. was used for dry weight determinations. Dry weights of tissues from birds receiving identical treatment were averaged and this average was used to calculate the Q_{O_2} for that particular tissue.

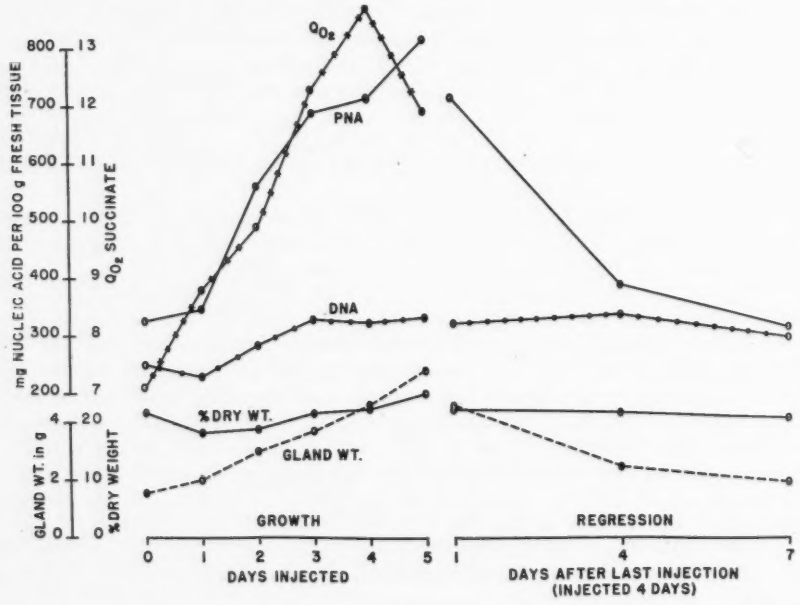

FIG. 1. Graphic representation of changes taking place in pigeon crop gland during the injection and after the withdrawal of lactogenic hormone.

RESULTS AND DISCUSSION

The data presented in the graphs in Figure 1 show that the rapid growth of the pigeon crop gland elicited by pituitary lactogenic hormone is cor-

related with increases in the concentrations of DNA and PNA, and succinic dehydrogenase. The detailed data (Table 1) from which the graphs in Figure 1 were made show that appreciable variation exists between the measurements made on the tissues of animals receiving the same treatment. Despite these variations the day to day averages are believed to be real and not due to chance.

It seems evident that the growth and increases in PNA, DNA and succinic dehydrogenase result from the first reactions that the hormone initiates and sets in motion, or otherwise from reactions that the hormone reorients toward growth. It is reasonable to assume that the growth of the gland and the increase in tissue substances such as enzymes and nucleic acids result from the mobilization of building blocks due to the action of the hormone. Presumably certain of these available metabolites are enzymatically oxidized to supply energy for the growth processes which result in the synthesis of tissues and the eventual elaboration of the "crop-milk."

The PNA content of stimulated crop gland tissue increased as much as 150.5 per cent over the value for unstimulated tissue (Table 1). This value is to be compared to a maximum increase of 62 per cent reported by Novikoff and Potter (1948) for regenerating liver. The increase in PNA of crop gland parallels rather closely the increase in fresh gland weight and is correlated with the gradual increase in succinoxidase activity. Since regenerating liver undergoes rapid growth it is of interest to point out that Novikoff and Potter (1948) found a decrease in the activities of the succinoxidase system, malic dehydrogenase, cytochrome reductase and oxalacetic oxidase in regenerating liver as compared to normal liver. These results indicate that during rapid growth different enzyme patterns are present in regenerating liver as compared to crop gland tissue.

As a complement to the above investigation, a study was made of the decrease in PNA during the regression or involution of the highly stimulated gland. The results are given graphically in Figure 1. They show that the rate of regression in the absence of the lactogenic hormone approaches the rate of growth under the influence of the hormone, and the changes in PNA are closely correlated with both growth and regression.

The concentration of DNA showed a slight decrease on day 1 followed by an increase of 31 per cent on day 3 after which the level remained relatively constant even during regression (Figure 1). The small decrease in DNA during the first day of injection may be a result of hydration and production of new cytoplasm. In this connection it should be noted that the epithelium of the lobes of the crop gland can be divided into two fairly distinct layers. The superficial layer, because of its later function in the

TABLE 1. CHANGES IN THE CONCENTRATION OF NUCLEIC ACIDS AND SUCCINIC DEHYDROGENASE OF PIGEON CROP GLAND DURING GROWTH STIMULATED BY LACTOGENIC HORMONE AND DURING REGRESSION OF THE GLAND RESULTING FROM WITHDRAWAL OF THE HORMONE

Days injected	No. of birds	Total hormone inj.	Crop gland		Mg. nucleic acid per 100 grams fresh weight: Succ. dehyd.					
			Fresh weight	Dry weight % fresh wt.	DNA		PNA		Q_{O_2}	% change
					Mean	% change	Mean	% change		
		ml.	gms.							
0	8	0.00	1.53 (1.26–1.65)[1]	21.6 (20–23)	251 (147–410)		326 (198–433)		7.1 (5)[2] (3.2–10.3)	
1	7	0.5	2.01 (1.51–2.98)	18.1 (13.6–21.5)	229 (181–265)	−8.8	346 (237–411)	6.1	8.8 (3) (6.7–10.2)	24.0
2	6	1.0	3.03 (1.68–4.8)	18.8 (16.7–21.3)	284 (227–344)	13.1	562 (411–737)	72.4	9.9 (4) (5.8–15.4)	38.9
3	7	1.5	3.67 (2.7–5.1)	21.7 (18.6–24.0)	328 (269–392)	30.6	690 (371–817)	111.7	12.3 (3) (11.0–13.9)	73.1
4	7	2.0	4.66 (2.9–7.6)	22.2 (19–26)	322 (271–357)	28.3	715 (588–789)	114.2	13.7 (6) (9.7–18.4)	78.4
5	8	2.5	5.82 (4.95–6.66)	25.0 (21–29)	331 (259–389)	31.8	817 (526–969)	150.5	11.9 (3) (9.9–16.3)	67.4
4 (1)[3]	7	2.0	4.66 (2.9–7.6)	22.2 (19–26)	322 (271–357)	28.3	715 (588–789)	114.2		
4 (4)[3]	4	2.0	2.47 (1.8–3.4)	21.8 (19.7–23.5)	338 (307–360)	34.7	389 (375–419)	19.3		
4 (7)[3]	4	2.0	1.96 (1.7–2.1)	20.7 (18.4–23.3)	298 (271–336)	18.7	316 (286–334)	3.1		
"Crop milk"	6	2.5		31.0 (29–32)	154 (130–191)		719 (544–951)		13.3 (2) (11.3–15.1)	

[1] Ranges.
[2] Figures in parentheses indicate number of determinations.
[3] Injected 4 days and killed on days 1, 4 and 7 after last injection.

formation of "crop milk," may be termed the "nutritive" epithelium. The deeper layers of cells in the region of the basal membrane can be termed the "proliferating" part of the epithelium. In the unstimulated crop gland these two layers are not as distinct as they are in the crop on the fourth or fifth days of injection. The changes in the epithelium under the influence of the lactogenic hormone are very much like those described during natural development (Beams and Meyer, 1931). A study of histological sections of the glands indicated that the increase in DNA occurred during the second and third days of injection at the time of greatest mitotic activity.

The "milk" found in the lumen of the crop is characterized for the most part as being composed of intact cells laden with fat droplets. The nuclei appear degenerate. Analyses were made of this "crop milk." The succinic dehydrogenase activity and PNA content were essentially the same as the value for crop gland tissue after 4 days of injection (Table 1). The DNA content of the "milk," however, was only about one-half as great as was found in the glands after 4 days of hormone. This observation is correlated with the degenerate nuclei found in the "milk" cells. The insoluble material obtained by centrifuging a homogenate of "milk" contained the succinic dehydrogenase activity. Thus, as has been found for tissues in general, the succinic dehydrogenase of crop gland "milk" is associated with insoluble particles.

SUMMARY

The pigeon crop gland when stimulated by injection of pituitary lactogenic hormone undergoes a rapid increase in weight. This growth is accompanied by an increase in the concentration of pentose nucleic acid and in the activity of succinic dehydrogenase. Desoxypentose nucleic acid increases to a much smaller extent. The levels of PNA and succinic dehydrogenase activity are as high in the "milk" as in the glandular tissue from which the "milk" is derived. When the hormone is withdrawn, the gland regresses at essentially the same rate as it grows under the influence of the hormone, and a decrease in PNA accompanies the regression.

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MECHANISMS OF DESOXYCORTICOSTERONE ACTION: RENAL EFFECTS OF THE WATER-SOLUBLE GLYCOSIDE

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STUDIES of desoxycorticosterone glycoside (DCG) in human subjects (Green, Johnson, Bridges, Lehmann and Gray, 1950) indicated that the effect on sodium excretion was the resultant of two contrary actions. Decreased tubular water reabsorption by increasing urine volume tended to be associated with a rise in sodium excretion. This tendency was opposed by a relative increase in tubular sodium reabsorption, manifested most noticeably by a fall in urinary sodium concentration.

The object of the present studies was to determine the extent to which these actions were modified by dosage and rate of administration, by salt-loading and by hypophysectomy.

METHODS AND MATERIALS

The studies were conducted on a series of 20 previously normal mongrel dogs. The weights of the animals varied between 7.5 and 20 kgm. Approximately equal numbers of males and females were represented. Surface area was calculated by the formula: $W_{kgm.}^{2/3} \times 0.107 = m^2$.

The experimental procedures were carried out under Dial urethane (0.7 cc/kgm intraperitoneally) or chloralose anesthesia (60 mgm./kgm. intravenously). Blood pressure, glomerular filtration rate, renal plasma flow and plasma and urine sodium concentrations were determined by standard methods as indicated in an earlier communication (Green and Farah, 1949).

Basal renal functional values were determined for each animal during 2 or more 30-minute control clearance periods. The effects of subsequent DCG administration on water and sodium excretion were studied in relation to changes in filtration and reabsorption rates. The rate of tubular water reabsorption was estimated by subtracting the urine excretory rate from the glomerular filtration rate. The rate of tubular sodium reabsorption was calculated as the difference between the urinary sodium output and the sodium filtration rate. The latter value was obtained by multiplying the glomerular filtration rate by the plasma sodium concentration.

The first group of experiments consisted in the administration of single intravenous DCG doses of 60 mgm. per square meter to 10 animals. The effects of the drug were studied during the succeeding four 30-minute clearance periods. The mannitol-PAH sustaining solutions were made up to isosmoticity with glucose-water solutions in four experiments, with sodium chloride solutions in another four, and to twice isosmoticity with sodium chloride solutions in the remaining two.

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A second group of experiments was conducted on eight animals infused with intravenous 0.86 per cent sodium chloride solution at the rate of approximately 1.66 cc. per minute per square meter throughout the period of observation. DCG was infused continuously at the rate of 20 mgm. per square meter per hour for 2 hours in three of the animals and for six hours in two. The three remaining animals received sodium chloride solution only. Clearance measurements were repeated hourly on all animals for 6 hours after the completion of the control clearance periods.

A similar set of measurements was made on two acutely hypophysectomized animals which were given 0.86 per cent sodium chloride solution at a rate of 1.66 cc. per minute per square meter throughout the experiment and DCG in a total dose of 40 mgm. per square meter over a period of three hours. These animals were studied for an additional two hours following the administration of the test drug.

To provide a more uniform basis for comparison of various procedures, the data from each animal were calculated as percentages of the values determined during the control clearance periods. Group values were derived by averaging the individual percentage changes.

RESULTS

The effect of single DCG doses on filtration rate was variable. In a majority of instances a small reduction was observed. Continuous DCG infusions were followed by moderate elevation of filtration rate, which averaged twenty per cent during the first hour and subsequently regressed toward or below the preinjection values. The changes in renal plasma flow were of smaller magnitude, so that the filtration fraction usually increased slightly.

The water output was increased by single DCG doses to levels which varied from 50 to 300 per cent greater than the control values. The rise was due primarily to decreased tubular reabsorption. This effect was most marked and sustained during the simultaneous infusion of sustaining solutions made up either with glucose and water or with hypertonic sodium chloride solution (fig. 1, A and C). When isosmotic sodium chloride sustaining infusions were used, the increase in water output was transitory and less intense (fig. 1B).

The effect of single DCG doses on urinary sodium concentration varied markedly with the composition of the sustaining infusion. In the presence of glucose-water solutions, the sodium concentration dropped to an average level of approximately 40 per cent of the control value. In some instances, the concentration was reduced almost to the vanishing point. When the sustaining infusions were made up with sodium chloride, the urinary sodium concentration showed a smaller decline and reverted to or above control levels within two hours after DCG injection.

The sodium output reflected the relative magnitude of the actions of DCG on water output and sodium concentration. In the presence of glucose-water sustaining infusions, the relatively greater fall in urinary sodium concentration outweighed the increase in water output and a well-marked

decrease in sodium output resulted. When the sustaining infusions were made up with isosmotic sodium chloride solution, the two actions very nearly counter-balanced one another, with the production of a small net fall in sodium output most evident during the second hour after injection. When hypertonic sodium chloride sustaining infusions were used the increase in water output in the absence of a significant change in urinary

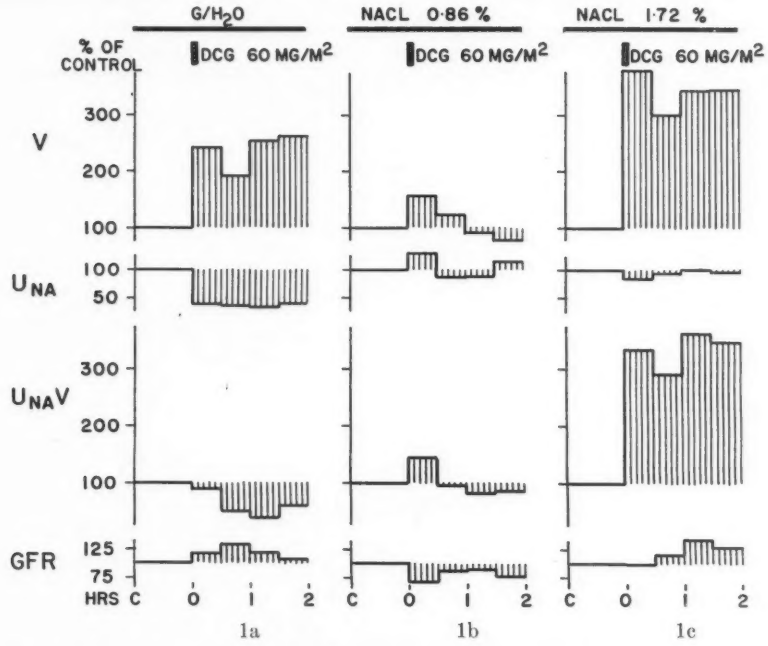

FIG. 1. Mean effects of single DCG doses on renal functions of groups of dogs simultaneously infused with mannitol-PAH sustaining solutions made isosmotic with glucose (1a) and sodium chloride (1b) and twice isosmotic with sodium chloride (1c). Values are expressed as percentages of the preinjection levels (C to 0 hours).

sodium concentration resulted in a rise in sodium output which paralleled the effect on water excretion.

The results of this group of experiments indicated that the action of single DCG doses was modified by the sodium load existing at the time of administration. This relationship was explored further by administering DCG continuously during the simultaneous infusion of sodium chloride. The results are perhaps best considered in relation to the changes in water and sodium excretion manifested by the non-DCG treated controls (fig. 2A). Continuous administration of isosmotic saline solution was followed

by a rise in water and sodium excretion which reached a maximum between the second and third hours. The elevation in sodium output was relatively greater than that of water due to increased urinary sodium concentration. With continuation of infusion, both water and sodium output receded from the maximum despite increasingly positive balances of the two substances. The reason for this cyclic behavior has been discussed previously (Green and Farah, 1949) and appears related primarily to changes

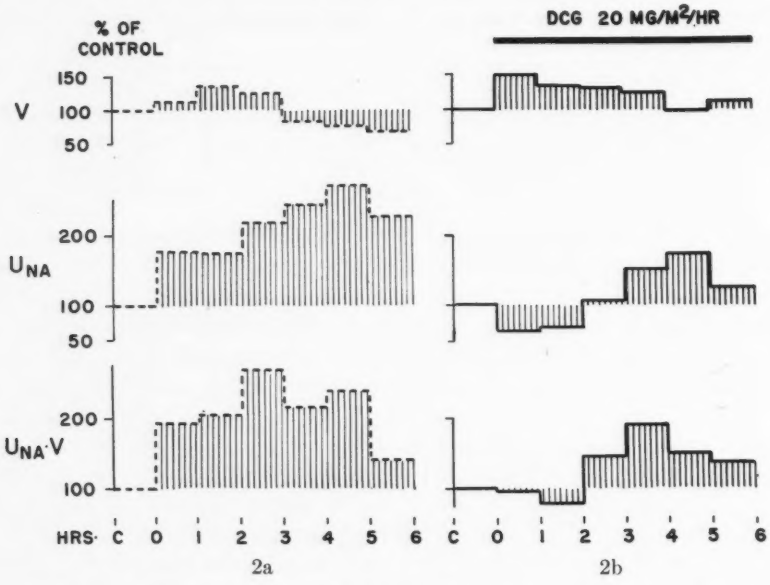

FIG. 2. Mean effects of continuous isosmotic sodium chloride infusions in control animals (2a) and in a group continuously infused with DCG (2b). Values are expressed as percentages of the levels determined during the control clearance periods (C to 0 hours).

in osmotic balance produced by excessive urinary loss of water relative to sodium until the concentration of sodium in the urine rises to approximate that of the infused solution.

DCG administration to animals similarly salt-loaded was followed by an immediate rise in water excretion, maximal during the first hour, with a subsequent decline despite continued DCG infusion (fig. 2B). The output of water remained above the preinjection value throughout the period of observation. The immediate effects on urinary sodium excretion consisted of a fall in both concentration and output, maximal during the first two hours of DCG administration. During the third and succeeding hours, the

urinary sodium concentration and output rose. Maximum values, which averaged nearly twice the control levels, were reached between the fourth and fifth hours.

Comparison of the group continuously infused with DCG with the control group showed that the behavior of both was essentially similar in pattern after the second hour. The net effect of continuous DCG infusion

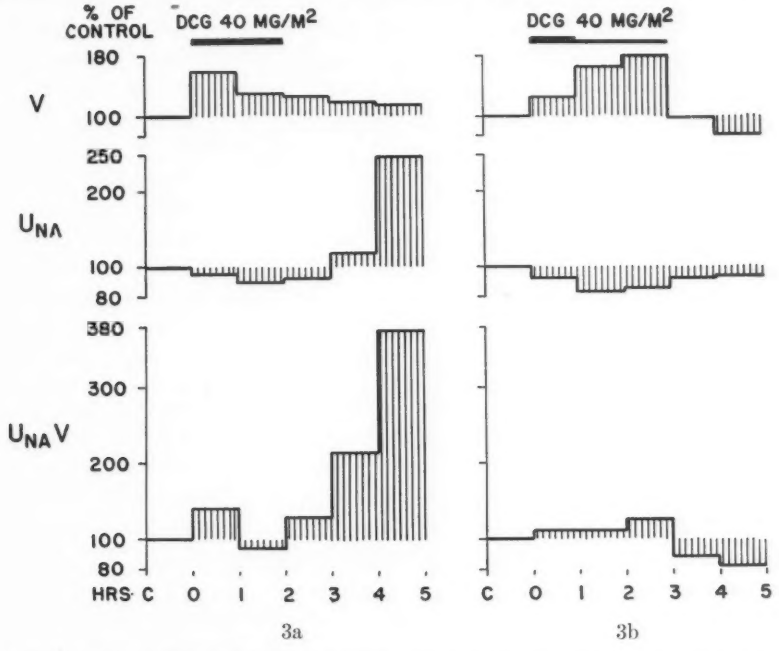

FIG. 3. Mean effects of 2 hour DCG infusions in normal animals (3a) and in a group hypophysectomized immediately prior to the period of observation (3b). Values are expressed as percentages of the levels determined during the preinjection clearance periods (C to 0 hours).

appeared to have been the displacement of the base line for water excretion upward and that for urinary sodium concentration and excretion downward. One result of this displacement was the development of a negative water balance in the DCG-treated group in contrast to the positive balance shown by the controls.

These results were interpreted to mean that the effects of salt-loading are to a considerable extent antagonistic to those induced by DCG administration; the net change in water and sodium excretion is the resultant of these opposing forces. This antagonism was more evident in animals to

which a 2 hour DCG infusion was given (fig. 3A). On its discontinuation a marked rebound occurred from the drug-induced depression of urinary sodium concentration and excretion which resulted in their elevation to levels two to four times greater than the preinjection values.

In view of the reciprocal relationship between the adrenal cortex and the posterior pituitary, the possibility was considered that the reversal of DCG effects during and after continuous infusions might involve the activity of hypophyseal factors. This possibility led to the trial of DCG infusions in acutely hypophysectomized animals (fig. 3B). In contrast to the effects noted in normal animals, DCG produced a continuous rise in water excretion throughout the period of administration. The increase in urine volume was sufficient to produce a small mean rise in sodium excretion despite a fall in urinary sodium concentration. When DCG infusion was discontinued the rebound in urinary sodium concentration and output which characterized the normal animal was absent. The water output fell to less than the preinjection value while the urinary sodium concentration failed to rise above the control level. As a consequence the net sodium output decreased.

DISCUSSION

A corollary to our findings that the effect of DCG on sodium reabsorption is inversely related to sodium load is the observation that the tubular reabsorption of sodium is a direct function of the sodium load in the adrenalectomized dog (Rommelt, Sartorius and Pitts, 1949). The combination of these data suggests that the deficiency of a DCA-like factor is responsible for the inability of the adrenalectomized animal to respond to sodium loading as well as to water loading.

Another adrenal cortical compound, 11 dehydro-17-hydroxycorticosterone, produces sodium loss in the normal dog (Thorn, Engel and Lewis, 1941) but produces sodium retention in the patient with Addison's disease (Forsham, Bennett, Roche, Reiss, Slessor, Flink and Thorn, 1949). Similarly, ACTH can produce either sodium retention (Forsham, Thorn, Prunty and Hills, 1948) or sodium loss (Sayers, Burns, Tyler, Jager, Schwartz, Smith, Samuels and Davenport, 1949). Some of these apparent discrepancies may have been due to differences in sodium load at the time the drug was administered.

Two possible mechanisms may account for the sodium rebound noted after cessation of DCG infusions in normal animals, and its absence following hypophysectomy. In the normal animal, the rebound may have been caused by increased release of the antidiuretic principle of the posterior pituitary gland. Findings in support of this possibility have been amply documented (Rommelt, Sartorius and Pitts, 1949; Gaunt, Birnie, and Eversole, 1949). An alternative hypothesis would involve the libera-

tion of an antagonist to DCG from the adrenal cortex through an action of the anterior pituitary gland. Both adrenal cortical extracts and ACTH are known to counteract the increased blood sodium produced by desoxycorticosterone acetate (Woodbury, Cheng, Sayers and Goodman, 1950), while 11 dehydro-17-hydroxycorticosterone increases sodium excretion in animals pretreated with desoxycorticosterone (Forsham, Bennett, Roche, Reiss, Slessor, Flink and Thorn, 1949). The latter hypothesis seems less likely since desoxycorticosterone has been reported to supress rather than stimulate the adrenocorticotrophic activity of the anterior pituitary (Sayers and Sayers, 1948). The data presented in this study do not exclude either of these hypotheses.

SUMMARY AND CONCLUSIONS

Single doses and continuous infusions of desoxycorticosterone glycoside (DCG) were administered to a series of dogs under various conditions of sodium load. The renal effects were studied in relation to changes in rates of filtration and reabsorption.

The most consistent and striking effect was the increase in water excretion, amounting from 50 to 300 per cent above control values. The rise was due to a decreased tubular reabsorption of water.

The second effect of DCG was a reduction in the urinary sodium concentration. The degree of change was a function of the sodium load. The per cent reduction in sodium concentration was more marked in glucose infused dogs than in animals which received isotonic sodium chloride solutions. When the animals were loaded with hypertonic sodium chloride solution the fall in urinary sodium concentration was prevented or reversed.

The effect on sodium output was the resultant of the changes in water output and urinary sodium concentration. Due to the relation of the latter factor to sodium load, output tended to fall in animals with a low sodium load, although urine water excretion was markedly increased. Conversely, sodium output increased in animals subjected to high sodium loads. When the load was imposed rapidly, as in the form of a hypertonic solution, the increase produced by DCG was immediate. When the load was imposed slowly, the increase was delayed for several hours, and preceded by a period of reduced sodium output. The direction and extent of the changes in output were attributable to corresponding alterations in tubular sodium reabsorption. The data indicate that the net influence of DCG on tubular sodium reabsorption is modified by the sodium load on the animal.

About one hour after discontinuation of DCG infusions sodium excretion increased markedly, although water excretion diminished. This sodium rebound was absent in hypophysectomized animals. Two possible mechanisms of this phenomenon have been discussed.

Acknowledgments

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TWO TYPES OF BASOPHILS IN THE ANTERIOR PITUITARY OF THE RAT AND THEIR RESPECTIVE CYTOPHYSIOLOGICAL SIGNIFICANCE

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INTRODUCTION

THE kresazan method of Romeis (1940), a combination of resorcin-fuchsin with azan, enabled him to differentiate between two types of basophils in the hypophysis of several species, including the rat. Later investigators, however, did not obtain encouraging results with kresazan on the rat's hypophysis (Brolin, 1945; Goldberg and Chaikoff, 1950; Gomori, personal communication). A new elastic stain, aldehyde-fuchsin, in combination with a modified azan technique, permits the clear-cut distinction between two classes of basophils in the pituitary of the rat (Gomori, 1950).

The present study was undertaken to establish further the distinct entity of these cell types, which will be referred to as beta and delta cells after the terminology of Romeis. Furthermore, attempts were made to elucidate their respective share in the production of the hormones generally attributed to the basophils of the anterior pituitary lobe: the follicle-stimulating hormone (FSH), thyrotrophin and the adrenocorticotrophic hormone (ACTH). For this purpose, groups of animals were subjected to experimental procedures, which are known to have an influence on the basophil cells and/or to modify the secretion of the aforementioned hormones.

MATERIALS AND METHODS

Seventy-nine young adult rats (200–300 gm.) of the Sprague-Dawley strain were used in this study. All animals were fed the same chow diet and kept at a constant temperature, unless otherwise specified.

Operations were performed under Nembutal anesthesia.

The optimal conditions of the aldehyde-fuchsin method as applied to the pituitary differ in some respects from those given in the original description by Gomori. Of the fixatives tried, Susa and Bouin (the latter with no more than 2% acetic acid) proved highly satisfactory, formol-sublimate gave poorer results, while formalin and Champy's fluid were found unsuitable. All pituitaries were double-imbedded in celloidin and paraffin and cut serially at 3μ in the horizontal plane. Reliable and elective staining of the

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beta cell granules with aldehyde-fuchsin within 5–10 minutes was obtained, if the solution was allowed to ripen for 72 hours and was not older than 4–5 days. The counterstain employed was essentially an azan technique with light green substituted for aniline blue. The result of this staining is the following: nuclei red, acidophil granules orange, beta cell granules violet to violet-blue, delta cell granules green to blue-green, cytoplasm of chromophobes unstained or pale grey-green.

Two thousand cells were counted in at least two representative sections from each gland according to the method of Rasmussen and Herrick (1922).

TABLE 1. PERCENTAGE OF CHROMOPHILS IN PITUITARY

Experimental group	Number of rats	Acidophils	Beta cells	Delta cells	Vacuolated	
					Beta cells	Delta cells
Normal males	20	40.83*±1.00** (33.85–48.85)†	2.77±0.17 (1.00–4.15)	5.86±0.18 (3.85–7.00)	0.02 (0.00–0.15)	0.04 (0.00–0.25)
Normal females	11	38.01±1.06 (29.55–41.35) −1.86††	2.43±0.28 (1.60–4.40) − 1.10	2.60±0.35 (1.15–4.95) −9.60	0.01 (0.00–0.05)	0.03 (0.00–0.10)
Castrated males	11	39.70±1.29 (32.05–47.30) −0.78	2.74±0.18 (1.65–3.90) − 0.12	see Fig. 1	0.03 (0.00–0.15)	see Fig. 1
Thyroidectomized males	11	14.75±5.46 (0.00–41.50) −4.78	0.28±0.09 (0.00–0.80) −11.24	see Fig. 2	0.00	see Fig. 2
Hemiadrenalectomized males exposed to cold	9	43.18±1.07 (38.65–47.75) +1.54	5.08±0.56 (2.35–7.80) + 5.50	7.42±0.27 (6.20–8.70) +5.03	0.36 (0.20–0.60)	0.34 (0.00–0.60)
DOCA-treated males	5	44.44±1.74 (38.10–47.95) +1.80	4.75±0.63 (3.35–6.50) + 4.63	8.59±0.62 (6.70–10.60) +6.67	0.06 (0.00–0.30)	0.03 (0.00–0.10)
Formalin-treated males	6	41.17±0.73 (37.85–43.40) +0.19	1.78±0.27 (0.96–2.50) − 3.09	9.90±0.91 (7.25–12.55) +6.95	0.01 (0.00–0.05)	0.12 (0.00–0.30)
Adrenalectomized males	6	46.95±1.92 (38.75–50.05) +2.59	2.20±0.18 (1.40–2.75) − 1.68	7.03±0.87 (4.35–10.85) +2.34	0.02 (0.00–0.10)	0.16 (0.00–0.50)

* Mean.
** Standard error ($S_{\bar{x}}$).
† Range.
‡ t-value of difference from mean of male control group.

EXPERIMENTS AND RESULTS

1. Controls

(*a*) *Males.* The results of the quantitative observations on the hypophyses of normal males are presented in Table 1. The pattern of distribution of beta and delta cells was found to be entirely different, though the two cells were often encountered next to each other. The beta cells are most numerous in the core of the gland, whereas the delta cells are most frequent in the anteromedian and dorsal parts of the lobe. Fig. 3 clearly shows the differences in the appearance of the individual beta and delta cells. The majority of the delta cells are rounded, while the average beta cell has irregular outlines, giving the impression of being compressed by the adjacent cells. The delta cells often have pyknotic or kidney-shaped

nuclei without prominent nucleoli, whereas the nuclei of the beta cells are mostly vesicular and provided with a large nucleolus. Occasional vacuolation is found among both the beta and the delta cells.

(*b*) *Females.* The only significant quantitative difference between the pituitaries of males and females was the considerably lower delta cell count in the latter (Table 1). The identical distribution, cytological appearance and similar percentage of the beta cells in the sexes deserves

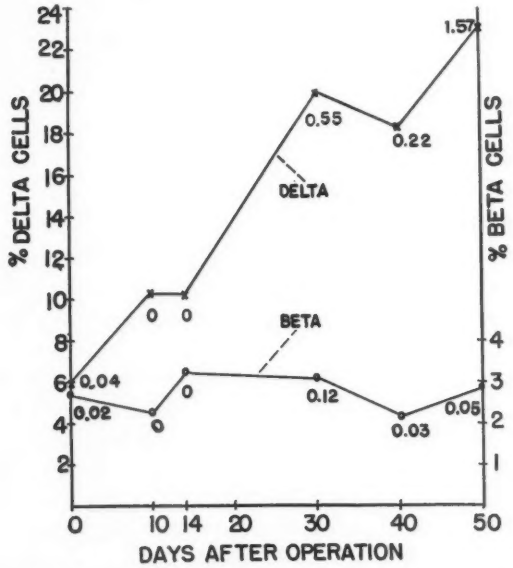

FIG. 1. Time curve of changes in the percentage of beta and delta cells after castration. The numerals along the curve in this and the following graph indicate the percentage of vacuolated cells.

emphasis. Though no substantial sex-difference in the acidophil count was encountered, the smaller size and sparse granulation of the acidophils in the pituitary of the female was found to be so conspicuous as to make it distinguishable from that of the male even under low power.

2. Castration.

The progressive increase in number and size of the basophils in the hypophysis of the castrated rat is one of the best-known and most striking experimental changes in this gland. It was of interest to examine, how the two classes of basophils are affected by castration.

Orchido-epididymectomy was performed on 11 males and the rodents

were sacrificed in groups of two or three at intervals of 10, 14, 30, 40 and 50 days after the operation. The observations made on their pituitaries are shown in Table 1 and Fig. 1. They can be summarized as follows: (a) A progressive augmentation of the delta cell count occurs. (b) The percentage of the beta cells remains unchanged. (c) The delta cells become hypertrophic, while the beta cells retain their angular, wedged-in appearance (Fig. 4 and 5). (d) A large scale formation of vacuolated "castration cells" is first observed among the delta cells of the 50-day group. The vacuolation of the beta cells stays within normal limits. (e) The acidophils show no significant changes.

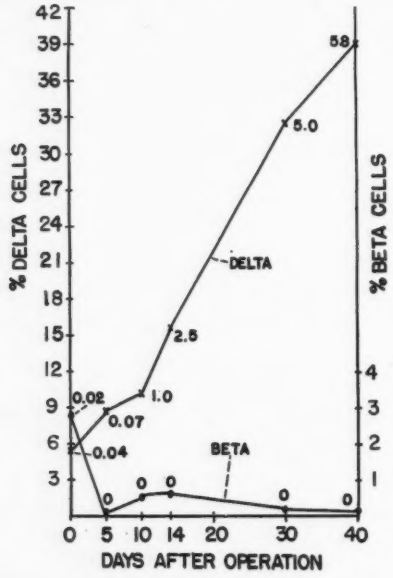

FIG. 2. Time curve of changes in the percentage of beta and delta cells after thyroidectomy.

3. Thyroidectomy

Changes similar to those seen after castration are known to occur in the pituitary of the thyroidectomized rat. However, the hypophyses of the thyroidectomized rodents can be distinguished by virtue of the earlier onset of vacuolation in the hyperplastic basophils and the degranulation or disappearance of the acidophils.

Eleven thyroparathyroidectomized male rats were sacrificed in groups of two or three at intervals of 5, 10, 14, 30 and 40 days after the operation.

The results of the cell counts made on their pituitaries are demonstrated by Table 1 and Fig. 2 and can be summed up thus: (a) A rapidly progressing hyperplasia and vacuolation of the delta cells takes place. (b) An almost complete disappearance of normally granulated beta cells is found beginning as early as 5 days after the operation and constitutes an additional characteristic distinguishing mark between the pituitary in castration and thyroidectomy. In some of the rats a relatively large number of pyknotic beta cells is encountered. (c) While the acidophils are absent or markedly reduced in most of the animals, their occasional persistence indicates incomplete removal of the thyroid in these cases (Lebedewa, 1936; Purves and Griesbach, 1947). (d) The regression of the beta cells is present even when the thyroidectomy was subtotal, as judged by the behavior of the acidophils, and thus proves to be a more sensitive sign of thyroxine deficiency than the disappearance of the acidophils. The structure of the pituitary after thyroidectomy is shown in Fig. 6.

4. Hemiadrenalectomy and subsequent exposure to cold

A combination of these two experimental procedures was used by Finerty and Briseno-Castrejon (1949) to evoke increased secretion of ACTH.

Nine sinistrally adrenalectomized male rats were exposed to +2° C. for 21 days. The histological findings in the hypophyses (Table 1) are as follows: (a) There is no statistically significant change in the number of acidophils. (b) The beta cells are significantly more numerous. Many of them are enlarged, spherical and more or less degranulated (Fig. 7). (c) There is an augmentation in the percentage of the delta cells. (d) A higher incidence of vacuolated cells is encountered both in the delta and the beta group.

The mean weight of the removed left adrenals in these animals was 21 mg., that of the right adrenals at autopsy 33 mg. The acini of the thyroid glands were found to be lined by tall columnar epithelium.

5. Administration of desoxycorticosterone acetate (DOCA)

Prolonged treatment with large doses of DOCA leads to adrenal cortical atrophy (Selye and Dosne, 1940; Carnes *et al.* 1941), which at least partly seems to be due to a decreased output of ACTH (Greep and Deane, 1947; Sayers and Sayers, 1948).

Five male rats received daily subcutaneous injections of 10 mg. DOCA in 0.5 cc. peanut oil for 21 days.[2] Higher than average counts were found

[2] DOCA was generously supplied by Mr. F. F. Houghton of Ciba Pharmaceutical Products, Summit, N. J.

in all three classes of pituitary chromophils, the increase in the beta and delta group being statistically significant (Table 1). The characteristic clusters of fully granulated beta cells observed in these hypophyses are shown in Fig. 8.

Significant weight losses as compared to the controls were found in the adrenals (19 vs. 42 mg.), the seminal vesicles (358 vs. 582 mg.) and the thymus (400 vs. 548 mg.) of the members of this group.

6. Acute stress

In order to examine the reaction of the two types of basophils to acute stress, which is known to have a profound influence on the pituitary-adrenal axis, six male rats received daily subcutaneous injections of 0.5 cc. 4% formalin for 3 days. As it is evident from Table 1, there is no change in the acidophils, while there is a probably significant drop in the number of beta cells and a highly significant rise in the percentage of delta cells. No definite qualitative changes were seen in any of the three chromophils.

7. Adrenalectomy

Sayers and Cheng (1949) reported a spectacular drop in the ACTH content of the rat's pituitary 24 hours after adrenalectomy, but failed to find corresponding histological alterations in the gland stained by Koneff's modification of the azan method. Since this does not differentiate between beta and delta cells, it seemed indicated to repeat their morphological investigations with the new staining technique.

Six male rats were adrenalectomized and sacrificed 24 hours later. The acidophil and delta cell count was higher, that of the beta cells somewhat lower than the mean of the controls, but the statistical significance of these findings could not be established (Table 1), nor did the cells display any unequivocal qualitative deviations from those of the normal rats.

PLATE 1. DESCRIPTION OF FIGURES

All photomicrographs are taken with a green filter and at a magnification of 900×.

FIG. 3. Pituitary of normal male rat. The arrows point at delta cells. The beta cells are dark, the acidophils grey.

FIG. 4 and 5. Pituitary 30 days after castration. The arrows point at beta cells. Note presence of acidophils and enlargement of delta cells. A vacuolated delta cell is seen near the left margin of Fig. 5.

FIG. 6. Pituitary 40 days after thyroidectomy. Acidophils and beta cells are absent. Several vacuolated delta cells.

FIG. 7. Pituitary of a hemiadrenalectomized-cold exposed rat. The arrows point at spherical, enlarged and partially degranulated beta cells, some of which are vacuolated.

FIG. 8. Groups of fully granulated beta cells in the pituitary of a DOCA-treated rat.

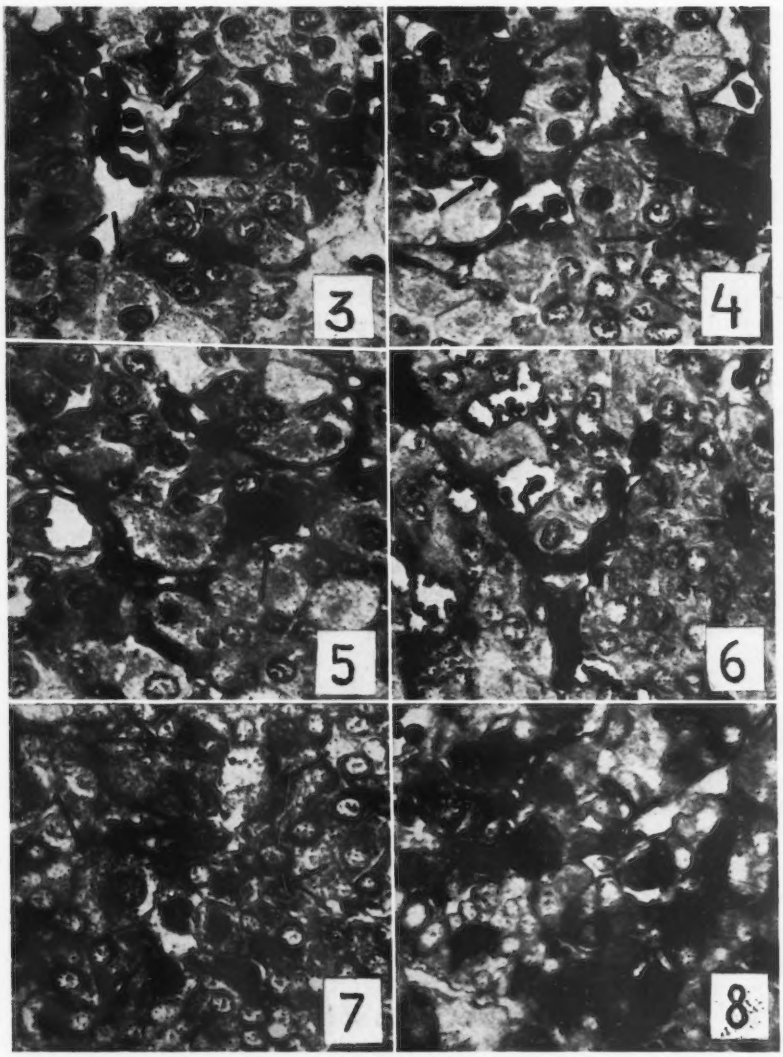

PLATE 1

DISCUSSION

Are the beta and delta cells independent cell types? Differences in distribution, cytological characteristics, percentage in the sexes and the obvious dissimilarities in their response to the experimental procedures applied justify an affirmative answer to this question.

In spite of the difficulties encountered when attempting to correlate morphology and function in the pituitary, some cytophysiological conclusions can be drawn from the observations reported.

There is a gratifying unanimity among the investigators of hypophysial histophysiology in attributing the elaboration of FSH to the basophils. Two of the most impressive arguments in favor of this view are the lower FSH content of the female pituitary (Evans and Simpson, 1929b), which is poorer in basophils, and the increase in gonadotrophic potency of the castrated rat's hypophysis (Engle, 1929; Evans and Simpson, 1929a), which develops *pari passu* with the hyperplasia and hypertrophy of the basophil cells. Since we have found that the lower total basophil count of the female pituitary is entirely due to the delta cells, and, in addition, the castration changes take place exclusively in the delta cells, the conclusion seems legitimate that the delta basophils are responsible for the formation of FSH. This is borne out by the observations on the delta cells in other experimental groups, viz. (a) their augmentation in the DOCA-treated animals, which probably represents an attenuated castration effect and harmonizes with the atrophy of the seminal vesicles, and (b) their higher percentage in the rodents subjected to stress, which can be interpreted as the morphological basis of Herlant's (1943) disclosure that alarming stimuli lead to an increase in the gonadotrophic potency of the pituitary.

Inference from the sequels of thyroidectomy in the pituitary as to the site of formation of thyrotrophin is rendered difficult by the occurrence of changes in both the basophils and the acidophils and the divergent findings on the thyroid-stimulating power of the pituitary after thyroidectomy. The ingenious studies of Griesbach and Purves (1945), however, afforded good evidence that the hyperplasia of the basophils is the sign of increased thyrotrophin production and that the basophil cells are the source of this principle. This conclusion is in accord with the majority of earlier pertinent observations (reviewed by Adams, 1946). Since it was found in this study that the augmentation of basophils after thyroidectomy is due to a rise in the delta cell count, whereas the beta cells regress, it is logical to infer that the formation of thyrotrophin takes place in the delta cells. Furthermore, the higher number and frequent vacuolation of the delta cells in the hemiadrenalectomized-cold exposed animals finds its explanation in the increased thyrotrophin output in this group, which was evidenced by the tall columnar epithelium of the thyroid follicles. Brolin (1945) has previously shown that prolonged exposure to low environmental temperatures enhances the secretion of thyrotrophin in rats and leads to hyperplasia and vacuolation of the basophils in the hypophysis.

The site of elaboration of ACTH is an unsettled problem. Finerty and Briseno-Castrejon (1949) credit the acidophils for its formation, as they found their number increased in hemiadrenalectomized rats kept at low environmental temperatures, whereas transplantations of different parts of the bovine and porcine hypophysis have indicated that the portion rich in basophils contain more of the hormone (Smelser, 1944; Giroud and Martinet, 1948). The latter observations are in better agreement with the evidence offered by human Cushing's syndrome.

It is of interest to examine, how the consideration of two independent varieties of basophils contributes to the solution of this moot question. For this purpose, the available data on the adrenocorticotrophic function of the pituitary under the conditions dealt with in this study have to be briefly reviewed.

The adrenocorticotrophic potency of the pituitary is (a) higher in the female and (b) remains uninfluenced by castration (Emery and Winter, 1934). (c) We are not aware of any data pertaining to the ACTH content of the pituitary after thyroidectomy. However, Deane and Greep (1947) summarized and confirmed earlier observations concerning the regression of the adrenal cortex in the athyroid state, and concluded that it is probably mediated by the pituitary. (d) Exposure to cold elicits an increase in the secretion of ACTH (Brolin, 1945). Previous adrenalectomy probably enhances this effect. (e) As already mentioned, DOCA is believed to bring about adrenal cortical atrophy partly through interfering with the release of ACTH. There are no data as to how prolonged treatment with large doses of DOCA affects the amount of stored pituitary ACTH. (f) Acute stress leads to a moderate and (g) adrenalectomy to a severe depletion of ACTH from the hypophysis (Sayers and Cheng, 1949).

It remains to discuss, how the reported morphological changes in each of the three classes of pituitary chromophils fit in with these data. (a) *Delta cells.* The discrepancies between the behavior of the delta cells and the adrenocorticotrophic function of the pituitary in the normal females, the castrates and the thyroidectomized animals is obvious. Generally speaking, their higher number in any of the groups can be correlated with an increased formation of either FSH or thyrotrophin. It seems unwarranted to postulate any connection between the delta cells and the elaboration of ACTH. (b) *Acidophils.* Our observations failed to give support to the contention that there is a definite positive correlation between changes in the acidophils and in the formation of ACTH. The amount of acidophilic granular material in the female pituitary wiht its higher adrenal-stimulating capacity is distinctly lower than in that of the male. Furthermore, no significant change in the acidophils followed any of the experimental procedures, which were designed to affect the secretion of ACTH. Only in

the group of the castrates and the thyroidectomized animals do the data on the behavior of the acidophils and the adrenocorticotrophic function harmonize. (c) *Beta cells.* There is a better agreement between the variations of the beta cells and the fluctuations in the adrenocorticotrophic activity of the hypophysis, than there was found with regard to the other two classes of cells. The parallelism is satisfactory in the castrates, the thyroidectomized and the formalin-treated rats, and especially the hemiadrenalectomized-cold exposed group, where the changes of the beta cells are highly suggestive of hyperfunction. The beta cells in the female are the only cell type, which is neither quantitatively nor qualitatively inferior to its counterpart in the pituitary of the male. The significance of the high number of fully granulated beta cells in the DOCA-treated rats is not clear. DOCA was expected to have a depressive influence on the cells forming ACTH, similar to that of ACTH itself on the basophils (Koneff, 1944). The latter observation is a good argument in favor of the view that ACTH is formed by the basophil cells. It may be, however, that DOCA interferes only with the release of ACTH from the pituitary and not with its formation. Cheng, Sayers and Sayers (1949) have shown that the outpouring of ACTH from the hypophysis after adrenalectomy can be partially prevented by DOCA. If merely the release of a hormone is blocked it is conceivable that a higher number of the cells that are its source would be found, as the discharge of granules and return to the chromophobic state does not take place in them. In the adrenalectomized group the beta cells, as well as the other two cell types, fail to show changes which could be interpreted as the morphological equivalent of the evacuation of ACTH from the hypophysis.

It can be concluded that the fundamental difficulties in evaluating the histophysiological significance of morphological findings in the pituitary have not yet been surmounted as regards the site of formation of ACTH and no cogent concordance between the changes in the secretion of this hormone and the behavior of any of the three cell types could be established. Nevertheless, the beta cells remain as a possible source of ACTH and their existence has to be taken into consideration in further studies which are required to elucidate this problem.

SUMMARY

Two types of basophils (beta and delta cells) can be distinguished in the pituitary of the rat by means of a new staining technique (aldehyde-fuchsin combined with a modified azan method). They show characteristic differences in number, distribution, cytological appearance and in their reaction to experimental procedures designed to bring about changes in the secretion of trophic principles (castration, thyroidectomy, hemiadren-

alectomy followed by exposure to cold, treatment with DOCA, acute stress and adrenalectomy). The delta cells are less numerous in the female. They become progressively hyperplastic and vacuolated after castration and thyroidectomy. Higher delta cell counts were found in the rats subjected to hemiadrenalectomy and subsequent exposure to cold, to acute stress and to prolonged treatment with DOCA. The beta cells are equally numerous in the sexes, remain unaffected by castration and tend to disappear after thyroidectomy. They are hyperplastic, enlarged and partially degranulated in the hemiadrenalectomized-cold exposed group. A high number of fully granulated beta cells is encountered in the DOCA-treated rats. The cytophysiological significance of these findings is discussed. It is concluded that the delta cells are the most likely source of FSH and thyrotrophin, while the possible role of the beta cells in the formation of ACTH requires further investigation.

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NOTES AND COMMENTS

PITUITARY-COMB RELATIONSHIP

Several investigators have made decidedly advanced steps in the determination of the relationship between pituitary and comb, and between comb and testes. The accepted relationship may be expressed as follows, viewing the comb as a passive receptor only.

$$\text{pituitary} \leftrightharpoons \text{testes} \rightarrow \text{comb}$$

Within the past few years Zawadowsky (1936), Marlow (1948), Hoskins and Koch (1939), and others have extended this relationship, which is expressed below:

$$\text{pituitary} \rightleftarrows \text{testes} \rightleftarrows \text{comb}$$

Hoskins and Koch suggested that a triangular reciprocal relationship might exist and that all unknown factors should be investigated in order to understand this cycle of sex physiology. This triangular relationship is expressed as follows:

$$\begin{array}{ccc} & \text{pituitary} & \\ \swarrow\nearrow & & \nwarrow\searrow \\ \text{comb} & \leftrightharpoons & \text{testes} \end{array}$$

The direct relationship of pituitary to comb as expressed by

$$\text{pituitary} \rightarrow \text{comb}$$

is the point in question in this communication.

EXPERIMENTAL PART

One hundred pounds of heads from young cockerels were removed directly upon killing, frozen immediately, and kept frozen until time for removal of the pituitary. This poundage represented about 900 cock heads. The cockerels were a cross between single comb white leghorns and Rhode Island reds.

Baby chicks one month of age were caponized bilaterally. They were allowed ten days of recovery and injected as indicated below.

The pituitaries were removed from the frozen heads and transferred to a weighed bottle and again weighed. Since 20 capons were to be tested daily as experimentals, 20 pituitaries were placed in each bottle. These were kept in the freezing compartment of a refrigerator and removed, bottle by bottle, daily as needed. These were ground with a flattened glass stirring rod using sand and with addition, in small portions, of 0.9% saline during grinding. The final saline extract, sand and cell fragments were transferred quantitatively to a centrifuge tube and centrifuged for five minutes at 1800 r.p.m. The protein suspension was decanted, the tube washed out, and the whole made up to a volume of ten ml. This allowed an injection of 0-5 ml. per chick, per day, equivalent to one pituitary. Since an equivalent of 108 mg./day was injected on the average, each chick received 5.4 mg. av/day of fresh tissue for the eight days. This was injected intramuscularly immediately after preparation. This procedure continued, using frozen samples each day for eight days. At this time the dosage was increased to two pituitaries per day per bird. An equivalent of 184 mg./day was injected, each of the 20 birds receiving 9.2 mg. equivalent of fresh pituitary tissue. This made a total injection of 42 pituitaries or 210.4 mgs. (average wt. of a pituitary was 5 mg. per bird) over 25 days period.

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TABLE 1

	Average gain					"t" value*		
	Wt.	Comb		L/Wt.	H/Wt.	L	H	Signif.
		L	H					
Experimentals	666	9.8	6.1	.0146	.0092	2.606	3.043	2.518 to 2.831
Controls	657	5.9	3.5	.0090	.0054			
Experimental (Slips)	549	25.0	15.0	.0456	.0274			
Control (Slips)	521	24.6	11.7	.0473	.0224			

* See Fisher and Yates "Statistical Tables for Biological, Agricultural and Medical Research" (p. 30).

The above results were computed on the average gain in weight, in length, and height of comb after rejecting all apparent "slips." (It might be said here (Table 1) that even the "slips" showed a marked increase of comb height and a slight increase in comb length after receiving the pituitary extracts.) It can readily be seen that the gain in height of comb is highly significant and the increase in comb length is statistically significant.

The capons mentioned above were held over several months until the comb changes had completely stabilized. Extracts of bovine pituitaries made in the same way as above, were injected, using increased doses as the birds were much larger. An average of 139 mg. of fresh pituitary was injected daily per bird for 28 days. Comb measurements were made weekly. No change in comb length or height was observed.

It is generally accepted that the adrenals are capable of producing androgens. The question here might well be raised that the pituitary stimulates the androgenic zone of the adrenal sufficiently to cause increased comb growth of the capons. Accordingly more chickens were caponized, and after sufficient recovery time was allowed, 0.5 mg. of purified ACTH (Armours) in saline was injected daily for 14 days. Comb measurements and body weights were recorded before, during, and at the close of the experiment. A summary of results is recorded below (Table 2):

TABLE 2

Animals	Body weight, gms.	Average gain		
		Body weight	Comb	
		gms.	Length, mm.	Height, mm.
Experimentals	785	285	0.3	2.0
Controls	797	274	0.0	1.3
Difference	−12	+11	+0.3	+0.7

A second experiment was performed on these same capons with ACTH of another sample (Searle) giving 5 mg./capon/day. The results are identical with those in the table above. It can be seen that there is no difference between the experimentals and controls.

DISCUSSION AND CONCLUSIONS

The increase in size of the comb under above conditions could conceivably be due either to (a) direct influence of an increased quantity of a pituitary substance(s) from a chick pituitary or to (b) the influence of ACTH of the pituitary upon the androgenic

zone of the cortex of the adrenals. The effect may be caused either by normal stimulation by the pituitary or by some functional aberration in the metabolism of the steroid compounds.

The results above show (a) a statistically significant increase in comb size by injections of saline extracts of the chick pituitary, (b) no change in comb size by use of ACTH, nor (c) by saline extracts of bovine pituitaries in large dosages. It is therefore believed that a direct relationship exists between quantity of some substance in the chick pituitary and the size of the capon comb. It is not believed to be due to adrenal stimulation by ACTH, unless the ACTH of the chick pituitary is species specific.

The author is indebted to Youngbloods Poultry Processing Company, Waco, Texas for furnishing the fresh frozen cock heads, and to Miss Marion Bucklin (Morgan) and Mrs. John Little for their timely aid in the preparation of this paper.

Acknowledgments

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THE EFFECT OF TARTRONIC ACID ON ABNORMAL FAT FORMATION FROM CARBOHYDRATE

Rats maintained on a natural fat-deficient diet form fat from carbohydrate to an abnormal degree as evidenced by respiratory quotients above 1.00 (Wesson, 1927, 1941; Wesson and Burr, 1931; Wesson and Murrell, 1933, 1934) under conditions in which rats on a complete diet average 0.91 as the maximum (Wesson, 1931). It has been shown that: (a) the high quotients are not due to intestinal fermentation (Wesson, 1933); (b) animal fats (Wesson, 1927; Wesson and Burr, 1931; Wesson and Murrell, 1933) and fruits and vegetables (Wesson, 1927, 1940) contain some factor which restores this carbohydrate conversion to the normal range; (c) the factor is not a saturated (Wesson and Murrell, 1933) or unsaturated (Wesson and Murrell, 1934) fatty acid (or ester) including the essential fatty acids (Wesson and Burr, 1931); (d) the factor is a saturated organic acid resistant to saponification (Wesson and Murrell, 1933, 1934); (e) the boiling of vegetables causes a loss of this factor (Wesson, 1940); (f) a continuance of the fat-deficient diet for more than a year leads to relatively low R.Q.'s during carbohydrate metabolism which again become higher than 1.00 under the influence of insulin but remain below 1.00 if the rats are first dosed with a concentrate of the factor for a few days before the insulin test (Wesson, 1941); and (g) there are indications that the glucose tolerance becomes abnormal in a number of the animals on the restricted diet at this later stage (possibly due to other deficiencies) (Wesson, 1941).

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In 1942 tartronic acid (hydroxymalonic) was found to be a constituent of the concentrate of the factor from lard.[1] It was tested at that time by the curative method for its activity in lessening fat formation from carbohydrate, with positive indications.[1] It has become possible only recently to test these earlier results by the use of the preventative method. The results by both methods are given in the following paragraphs.

PROCEDURES

Isolation and identification of tartronic acid. Each step in the separation was guided by the activity of the fractions. After saponification of the lard, the resultant solution was diluted, brought to pH 5.6, and extracted with a small volume of ether, then repeatedly with a large volume after strong acidification. The material taken up from the strongly acid solution was treated with aqueous K_2HPO_4, filtered, and the strongly acidified filtrate thoroughly extracted with ether. The residue left after vacuum distillation of the solvent was treated with acetic anhydride and sodium acetate, the excess anhydride decomposed with water, the solution partly neutralized, and the acetylated compounds extracted with a small volume of ether. These were saponified and the acids extracted from the strongly acidified solution with a large volume of ether, steam-distilled, and removed from the strongly acidified distillate by means of repeated ether extractions. The ether was evaporated, leaving white crystals on the walls of the container. The melting point of these, after drying, was 157° C. with rapid gas evolution. The m.p. of tartronic acid is variously given as 156° to 160° C. with decomposition. As similarly prepared but not as pure products had given a low neutralization equivalent, a low molecular weight as determined by the depression of the fusion point of camphor, and a dried barium salt containing 59% Ba (theor. for $BaC_3H_2O_5$:53.9% Ba), identification was made. Additional confirmation was given by the Denigès (1909) color reaction.

Tests for activity of tartronic acid. Eight rats[2] were used for the curative and 12 for the preventative series.

The fat-deficient diet for both series was: white dextrin, N.F.V, 72%; casein,[3] 18%; salts,[4] 4%; ether-extracted, dried brewers' yeast,[5] 6%; and carotene, 0.05 mg. per rat per day.

The R.Q.'s were obtained as previously described (Wesson, 1941), except that half-hourly instead of hourly determinations were made.

In the curative series, 10 mg. tartronic acid (Petriew, 1878) per 100 gm. body weight day was fed for 3 successive days. This was administered by medicine dropper in 1% aqueous solution to rats giving R.Q.'s significantly above 1.00 during carbohydrate metabolism. A day later the tests were made again. In the preventative series 1 mg. tartronic acid (Bak, 1939) per rat per day was added (as 0.3% ether solution) to the fat-

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[1] Unpublished.

[2] From the Animal Farm, Biology Department, Harvard University. Those used for the preventative method were 150 gm. male "Hisaw" rats inbred from the Sprague-Dawley strain.

[3] "Vitamin-free," The Borden Company, 350 Madison Ave., New York.

[4] For the curative method, Osborne and Mendel's, Harris Laboratories, Tuckahoe, New York; for the preventative, a modified Osborne and Mendel mixture (Wesson, 1932), General Biochemicals, Inc., Chagrin Falls, Ohio.

[5] For the curative method, Harris's; for the preventative, U.S.P.XII, Nutritional Biochemicals Corporation, Cleveland, Ohio.

deficient diet of 6 rats for the duration of the experiment, while 6 controls were given no tartronic acid. At the end of the 4 months, when the preventative series had been completed, the controls were given tartronic acid as in the earlier curative series, except that administration was by subcutaneous injection of an aqueous solution of the sodium salt (1% based on the acid).

RESULTS

By the curative method the average maximum R.Q. was lowered from 1.056 to 0.974, and the average R.Q. during the period of active carbohydrate metabolism was lowered from 1.004 to 0.952. In the preventative series (with tartronic acid) the maximum R.Q. averaged 0.947 and the mean R.Q. of active carbohydrate metabolism averaged 0.894, while for the controls (without tartronic acid) the corresponding values were 1.057 and 0.975. The controls, after treatment with tartronic acid, now gave the values 0.993 and 0.936, respectively. The action of tartronic acid in reducing fat formation from carbohydrate is apparent.

Of some confirmatory value is the fact that the control rats in the preventative series gained, in the course of the 4th month on the fat-deficient diet, an average of 18 gm. per rat more than those of the group receiving tartronic acid, and consumed 50 gm. per rat (12%) more food. In contrast, during the first two months the food intake and body weight were practically the same for the two groups.[6]

SUMMARY

The abnormal amount of fat formation from carbohydrate by rats maintained on a fat-deficient diet is markedly reduced by tartronic acid. This is shown by both the curative and preventative methods.

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[6] These results and those cited in the introductory paragraph suggest that further investigation should be undertaken with a view of determining whether a somatic deficiency of tartronic acid is of etiological significance in certain cases of human pancreatic diabetes (cf. Wesson, 1940, 1941). The findings of Rieben and Hastings (1946) that tartronic acid is present in liver and plant tissues, is exogenous in animals, and is not a metabolite, are of interest in this connection.

THE BIOLOGICAL ACTIVITY OF SODIUM TESTOSTERONE SULFATE

We have recently described the preparation of sodium testosterone sulfate (Holden, Levi & Bromley, 1949). The present report deals with experiments to test the androgenic effect of the compound following parenteral and oral administration to rats, and a single experiment on its excretion in the urine of a human subject following oral administration.

EXPERIMENTAL

Sodium testosterone sulfate and testosterone propionate were each dissolved in propylene glycol and administered subcutaneously and orally to castrated and to intact rats. Control animals received only propylene glycol in the same quantity and by the same route. At the end of the experiment the animals were killed with illuminating gas

TABLE 1. EFFECT OF SODIUM TESTOSTERONE SULFATE AND TESTOSTERONE PROPIONATE IN CASTRATED RATS BY SUBCUTANEOUS INJECTION OF ONE DAILY DOSE IN 0.2 CC. OF PROPYLENE GLYCOL: ANIMALS CASTRATED AT 40 DAYS AND TEST STARTED 30 DAYS AFTER CASTRATION. DURATION OF TEST, 15 DAYS. 4 RATS IN EACH TEST GROUP AND 2 RATS AS CONTROLS

Test	Daily dose, mgs.	Rat weight		Seminal vesicles		Prostate		Adrenals	
		Aver. gms.	Range gms.	Av. wt., mg.	Range, mgs.	Av. wt., mgs.	Range, mgs.	Av. wt., mgs.	Range, mgs.
Testosterone propionate	0.1	294	234–330	31	21–55	156	105–185	50	45–61
Sodium Testosterone sulfate	1.0	295	272–328	8	4–10	63	58–67	51	41–55
Controls	—	235	228–242	5.3	5–5.7	43	43–44	56	53–60

and the seminal vesicles, prostate and adrenals were dissected out and weighed: in the intact animals the testes were also weighed. The data in Table 1 show that in castrated rats subcutaneous injection of sodium testosterone sulfate in a dose ten times that of testosterone propionate has minimal androgenic effects. The weights of the adrenals are somewhat less than those of the controls.

Because sodium testosterone sulfate is very soluble in water it was thought that rapid excretion might account for the low androgenic activity. The total daily dose was therefore divided into three parts and administered four hours apart. The data in Table 2 show that divided dosage, subcutaneously and orally, produces only minimal androgenic effect. The adrenal weights are moderately decreased with all doses.

It was considered of interest to determine if, in spite of its lack of androgenic effect, sodium testosterone sulfate had an inhibitory effect on gonadotrophic activity of the anterior pituitary as evidenced by decrease in testicular weight following long term administration to intact animals. Table 3 records the results. It is quite obvious that, judged by testicular weight, sodium testosterone sulfate has no inhibitory action on the gonadotrophic activity of the anterior pituitary and again very moderate androgenic effects as judged by the slight increase in weight of seminal vesicles and prostate over that of the controls. The adrenals show no change in weight.

EXCRETION OF TESTOSTERONE IN URINE FOLLOWING ORAL ADMINISTRATION OF SODIUM TESTOSTERONE SULFATE

The failure of sodium testosterone sulfate to show significant androgenic activity suggested the possibility that the compound did not undergo the same metabolic transformation as that of testosterone and that it might therefore be possible to recover the compound in the urine after oral administration.

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TABLE 2. COMPARATIVE EFFECT OF SODIUM TESTOSTERONE SULFATE AND TESTOSTERONE PROPIONATE IN CASTRATED RATS BY SUBCUTANEOUS AND ORAL ADMINISTRATION: THE DAILY DOSE WAS DIVIDED INTO 3 PORTIONS AND EACH PORTION ADMINISTERED IN PROPYLENE GLYCOL 0.3 CC. FOR THE SULFATE AND 0.2 CC. FOR THE PROPIONATE. THE ANIMALS WERE CASTRATED AT 40 DAYS AND THE TEST STARTED 28 DAYS AFTER CASTRATION. DURATION OF TEST, 15 DAYS. 5 ANIMALS IN EACH TEST GROUP AND ONE GROUP OF 5 ANIMALS ACTED AS CONTROLS FOR ALL TESTS

Test	Daily dose	Rat weight		Seminal vesicles		Prostate		Adrenals	
		Aver., gms.	Range, gms.	Aver., mgs.	Range, mgs.	Aver., mgs.	Range, mgs.	Aver., mgs.	Range mgs.
Testosterone propionate	0.1 mg. subcut'ly.	245	202–270	58	38–70	233	195–263	41	36–49
Testosterone propionate	5.0 mg. orally	213	194–240	23	18–29	90	69–106	35	29–43
Sodium Testosterone sulfate	1.0 mg. subcut'ly.	225	202–244	4.7	3.3–6.2	51	38–80	43	32–62
Sodium Testosterone sulfate	5.0 mg. orally	214	198–250	6.4	3.3–8.2	51	38–60	37	31–44
Controls	—	240	200–256	6.0	3.8–8.6	37	33–42	46	41–56

TABLE 3. COMPARATIVE EFFECT OF SODIUM TESTOSTERONE SULFATE AND TESTOSTERONE PROPIONATE BY SUBCUTANEOUS ADMINISTRATION TO NORMAL RATS. AGE OF ANIMALS AT START, 35 DAYS. DURATION OF TEST, 30 DAYS

Test	Daily dose	Rat weight		Testes		Seminal vesicles		Prostate		Adrenals	
		Aver., gms.	Range, gm.	Aver., mgs.	Range, mgs.	Aver., mgs.	Range, mgs.	Aver., mgs.	Range, mgs.	Aver., mgs.	Range, mgs.
Testosterone propionate	1.0 mg. (9 rats)	185	164–234	1784	1406–2168	372	337–501	1424	1117–1681	37	29–46
Sodium testosterone sulfate	1.0 mg. (10 rats)	204	148–256	2656	2384–3092	150	58–270	603	226–880	35	24–48
Controls	— (8 rats)	182	145–208	2535	2081–3284	121	59–165	474	145–584	35	26–43

An excretion experiment was carried out in which 200 mg. of sodium testosterone sulfate was swallowed by a normal male, age 52. A sample of the 24 hour output of urine was extracted with butanol and the testosterone measured colorimetrically by the method described by Allen, Hayward and Pinto (1950) for the estimation of dehydroisoandrosterone. The total testosterone recovered amounted to 7 per cent of the testosterone ingested.

DISCUSSION

The sulfate ester of testosterone is quite stable to the hydrolytic action of barium chloride (Holden *et al.*, 1949). Should this stability be maintained in vivo it may indicate that the presence of a free hydroxyl is essential for androgenic activity.[1] In this connection Schapiro (1939) has shown that local application of methyl testosterone-triacetylglucuronate to the cock's comb produced practically no growth promoting effects. The observation that sodium testosterone sulfate is comparable to testosterone propionate in reducing the weight of the adrenals of castrated rats suggests the possibility of a dissociation of androgenic activity from action on the adrenal in the case of sodium testosterone sulfate. Finally, the observation that sodium testosterone sulfate is rapidly and almost completely metabolised raises the question of whether the metabolites recoverable in the urine differ from those which follow administration of testosterone.

SUMMARY

Sodium testosterone sulfate administered to castrated rats has very low androgenic potency when compared with testosterone propionate. Its effect in reducing the weight of the adrenals, however, is comparable.

In intact rats the sulfate, unlike the propionate, does not cause reduction in weight of the testes and neither compound, in the doses administered, affects the weight of the adrenals.

Seven per cent of the 200 mg. of sodium testosterone sulfate administered orally: in a single dose to a normal male was recovered in the following 24 hour urine.

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[1] The presence of a free hydroxyl in estradiol would also appear to be necessary since estradiol-17-monosulfate, which is also unhydrolysed by barium chloride, failed to show any estrogenic activity in ten times the effective dose of estradiol (unpublished results).

DOSE RESPONSE STUDIES OF THE EFFECT OF DESOXYCORTICOSTERONE ACETATE (DOCA) ON THE SODIUM EXCRETION OF ADRENALECTOMIZED RATS

An earlier paper by Dorfman *et al.* (1947) showed that radiosodium could be used to measure the rate of sodium excretion in adrenalectomized rats to detect small quantities of desoxycorticosterone and desoxycorticosterone acetate. The possibility of the development of a bioassay method based on these experiments was pointed out and in the present study the authors have endeavoured to obtain quantitative results to further this latter purpose.

METHOD

Albino rats of the Wistar strain bred in this laboratory weighing 130–150 gms. were used in these experiments. Bilateral adrenalectomy was performed by the dorsal route in one stage under ether anaesthesia and the animals maintained on a stock diet of rat cubes, containing 7.2 mg. K and 3.8 mg. Na/gm. original matter, and 1% saline during the four days before the start of the experiment. The rats were then placed in metabolism cages for the collection of urine; the complete voiding of the bladder was ensured by suprapubic pressure and the administration of a light whiff of ether. 1 mg. of sodium chloride containing 10 μc of Na^{24} Cl in 0.5 ml. water was injected subcutaneously at the start of the experiment. The radioactive sodium in the urine was measured in solution by a thin walled tubular Geiger counter (20th Century Electronics B. 12 type). This counter had a cathode thickness of 30 mg./cm.^{2} which effectively filtered out the soft β rays from S^{35} which is produced on neutron irradiation of the sodium chloride by a Cl (n.p.) reaction. DOCA (Organon Laboratories LTD) was injected subcutaneously in a solution of 0.5 ml. arachis oil. The same volume of this solvent was injected into the control rats.

RESULTS

1. Diurnal-nocturnal variation in the rate of sodium excretion

a). The rate of excretion of Na^{24} in intact rats supplied with tapwater was 0.16% of the initial dose per hr. by day and 0.37% per hr. by night.

b). The rate of excretion of adrenalectomized rats supplied with tapwater was 0.90% per hr. in the first period after injection and 0.44% per hr. in the second. This was independent of whether the first period was day or night.

c). The rate of excretion of adrenalectomized rats supplied with 1% saline was 0.70% per hr. diurnally and 1.70% per hr. nocturnally. This was independent of the time of injection.

Thus the variation in the rate of excretion of the intact rats on tapwater and of the adrenalectomized rats on 1% saline was similar, although the actual rate of the latter was much greater. However, when tapwater was substituted for 1% saline, the sodium excretion by adrenalectomized rats was always reduced about 10 hrs. after injection.

2. The effect of DOCA on the rate of Na^{24} excretion

All rats in the following experiments were adrenalectomized and supplied with 1% saline and the urine collected hourly.

The effect of DOCA was superimposed on the normal excretion pattern as shown for the control adrenalectomized rats in Fig. 1.

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The rate of sodium excretion of the injected rats for all doses of DOCA above 0.5 μg. fell below that of the controls for a certain time after injection and then rose sharply above the control rate. The action of the DOCA can be considered under two headings (i) the average depression of the rate of sodium excretion by the DOCA injected rats in an arbitrary interval of time after its injection, & (ii) the duration of this effect.

(i) The depression of the rate of sodium excretion varied with the dose of DOCA as

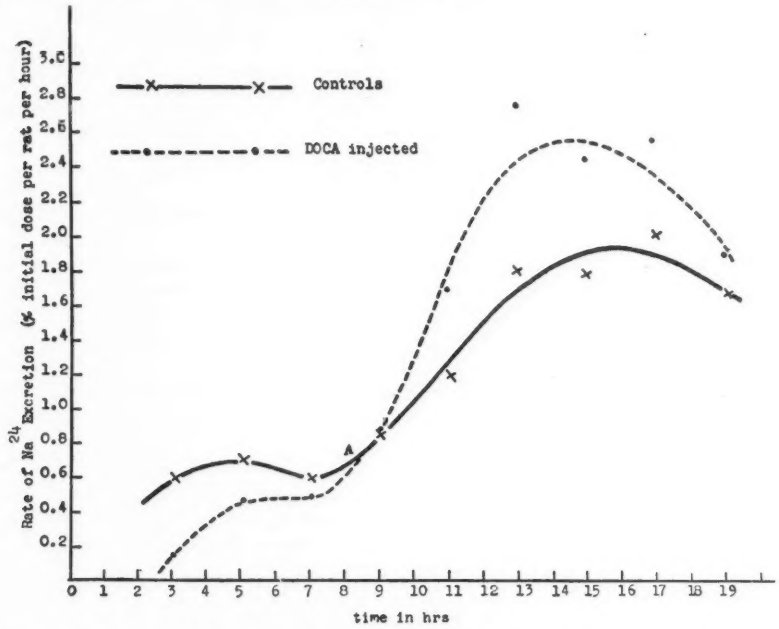

FIG. 1. 24 hr. sodium excretion pattern for control and DOCA injected adrenalectomized rats. Injection time of 2 mg. NaCl and DOCA was 9 A.M. represented by 0 hr. on abscissa. Dose of DOCA was 5 μg. 18 rats per group were used.

summarised in Table 1. The depression was expressed as the ratio of the average rate of excretion in the four hours after to that in the two hours before injection of DOCA. It should be noted that as a result of the diurnal decrease the ratio for the control rats was 0.80 instead of unity.

TABLE 1. THE RATE OF SODIUM EXCRETION OF ADRENALECTOMIZED RATS INJECTED AT 11 A.M. WITH VARIOUS DOSES OF DOCA. THE RESULTS ARE EXPRESSED AS A RATIO

$$\frac{Na^{24} \text{ excreted per hr. per rat 2 hrs. before injection}}{Na^{24} \text{ excreted per hr. per rat 4 hrs. after injection}}$$

Dose DOCA, μg. per rat	0	1	2	4	16	32	64
Ratio	0.80	0.63	0.61	0.54	0.41	0.28	0.31

The ratios are the mean of two or more experiments using 12 rats for each dose level.

(ii) The results of the investigation of the duration of the action of DOCA are given in Table 2.

TABLE 2. DURATION OF THE SODIUM-RETAINING EFFECT OF DOCA

Dose DOCA, μg. per rat	0.5	0.75	1.0	3.0	5.0	32.0
Duration time hr.	1.5	4	5	7	9.5	18

The duration of the sodium retaining effect was measured from the time of DOCA injection at 11 a.m. to that when the excretion rate again equalled that of the controls e.g. point A in Fig. 1. Sodium was given 2 hrs. before DOCA. The results were the means of two or more experiments using groups of 12 or 18 rats for each dose level.

OBSERVATIONS

The results shown in Tables 1 and 2 indicate that the rate of sodium excretion shows dependence on dose for a range of 0.5–32 μg.

A striking feature in the excretion pattern of the adrenalectomized rats injected with DOCA, as shown in Fig. 1, was the great increase in the rate of sodium excretion following the initial period of retention. This increase was such, that the rate of sodium excretion of the DOCA injected rats exceeded that of the controls for a time before gradually falling to the control level. The net effect of the DOCA, as measured over the total period, was one of sodium loss rather than retention. Thus in measurements of the action of DOCA in depressing sodium excretion, the time interval must be carefully chosen to avoid anomalous results.

Table 2 shows that for a dose range of 1–5 μg. the duration of the effect is linearly dependent on the actual dose, although a bioassay method based on this effect would be tedious in practice. A bioassay method based on measurements given in Table 1 would be the method of choice but the dose response curve shows that the experimental errors would have to be reduced to a minimum for reliable results. The main errors were considered to be:

a) Inaccuracy in the amount Na^{24} solution injected.
b) Loss of urine due to incomplete emptying of the bladder.
c) Loss of urine during collection.

A more serious disadvantage of the method was its comparative non-specificity, e.g., pitressin through its antidiuretic action also has a sodium retaining effect. Experiments are now proceeding in which K^{42} and Na^{24} in the urine are measured simultaneously. To eliminate the experimental errors mentioned above and to make the method more specific, the ratio of Na^{24}/K^{42} is taken as an index of the activity of DOCA.

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